Dissertation on

ESTIMATION OF CALCIUM, PHOSPHORUS, ALKALINE PHOSPHATASE AND INTACT PARATHYROID HORMONE IN VARIOUS STAGES OF CHRONIC KIDNEY DISEASE

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DEPARTMENT OF BIOCHEMISTRY

CHENNAI MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

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This is to certify that this dissertation entitled **"Estimation of Calcium, Phosphorus, Alkaline Phosphatase and intact Parathyroid Hormone in various stages of Chronic Kidney Disease"** is a bonafide work done by **Dr. M. Ganesha Pandian** in partial fulfillment of the requirements for M.D., Branch – XIII (Biochemistry) examination of **The Tamilnadu Dr. MGR Medical University** to be held in May – 2018. The period of study was from 2015 - 2018.

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This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai towards the partial fulfilment of the requirement for the award of M.D., Degree (Branch – XIII) in Biochemistry.

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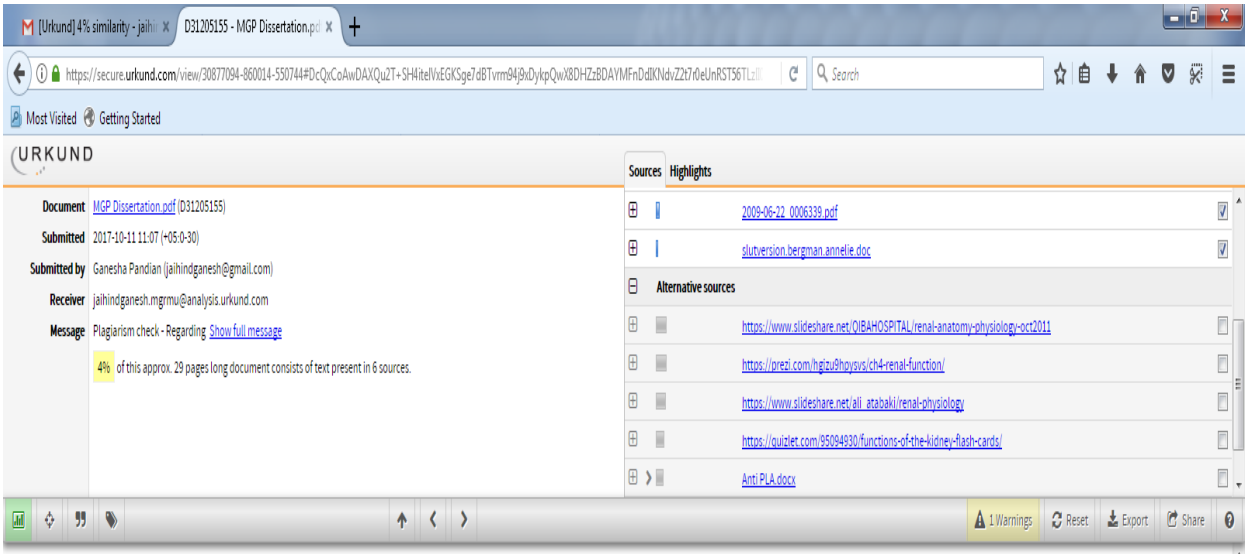

As kidney function declines, there is a progressive deterioration in mineral homeostasis with disruption of normal serum concentrations of phosphorus, calcium and changes in circulating levels of hormones like parathyroid hormone (PTH) and Vitamin D3. Beginning in CKD stage 3, the ability of the kidneys to appropriately excrete phosphorous load is diminished, leading to hyperphosphatemia. Kidneys fail to respond adequately to PTH, which normally promotes calcium reabsorption and phosphorus excretion. In addition, there is a down regulation of vitamin D receptor and resistance to the actions of PTH at tissue level causing secondary hyperparathyroidism. (1) Hyperparathyroidism plays a vital role in the excess morbidity and mortality in chronic kidney disease. As a result, patients are at increased risk of bone disease, extra osseous calcification, and death. (1) These changes probably begin early in the course of CKD, when Glomerular Filtration Rate (GFR) declines below 60 mL/min per 1.73 m 2 . (2) Cardiovascular disease accounts for 70% of all deaths in patients with CKD, with an overall mortality of 20% per year in patients on dialysis. (3) Left Ventricular Hypertrophy (LVH) is the most prevalent cardiac complication observed in CKD patients and is often associated with myocardial fibrosis, poor perfusion, and cell death. Hyperphosphatemia and hypercalcemia have been shown to promote calcification of the vasculature, myocardium, and cardiac valves. Vascular calcification, manifested in reduced vessel wall elasticity, increased intima media layer thickness is linked to LVH, and occurs with increased severity in dialysis patients versus non-CKD patients. (4) Vascular and soft-tissue calcifications are strong predicators of cardiovascular mortality among CKD patients.

Clinical care guidelines suggest that, at least annual testing and subsequent treatment of disorders of bone and mineral metabolism is essential early in the course of CKD when GFR is still 60 ml/min per 1.73 m 2 . But various investigations suggest that less than 25% of patients reach the target levels for intact parathyroid hormone (PTH) measurement or control. (5)

2. AIM & OBJECTIVES 1. To correlate serum intact parathyroid hormone, urea, creatinine, calcium, phosphorus and alkaline phosphatase in various stages of CKD and to compare the same with the control group. 2. To find the role of intact parathyroid hormone in the early diagnosis of mineral disturbances in CKD patients.

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ABBREVIATIONS

CKD	Chronic Kidney Disease
Ca	Calcium
Cl^-	Chloride
CT	Computed Tomography
cAMP	Cyclic Adenosine Mono Phosphate
CKD-MBD	Chronic Kidney Disease - Mineral Bone Disorder
CAD	Coronary Artery Disease
CVD	Cardiovascular Disease
CaR	Calcium Receptors
FGF-23	Fibroblast derived Growth Factor
GFR	Glomerular Filtration Rate
HCO_3	Bicarbonate
H^+	Hydrogen ion
HD	Hemodialysis
i PTH	Intact Para Thyroid Hormone
KDIGO	Kidney Disease Improving Global Outcomes.
KDOQI	Kidney Disease Outcomes Quality Initiative.
LVH	Left Ventricular Hypertrophy
MDRD	Modification of Diet in Renal Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
Na^+	Sodium
NKF	National Kidney Foundation
P	Phosphorus
SHPT	Secondary Hyper Parathyroidism

INTRODUCTION

1. INTRODUCTION

Chronic Kidney Disease (CKD) is an international public health problem affecting about 5–10% of the population. As kidney function declines, there is a progressive deterioration in mineral homeostasis with disruption of normal serum concentrations of phosphorus, calcium and changes in circulating levels of hormones like parathyroid hormone (PTH) and Vitamin D3. Beginning in CKD stage 3, the ability of the kidneys to appropriately excrete phosphorous load is diminished, leading to hyperphosphatemia. Kidneys fail to respond adequately to PTH, which normally promotes calcium reabsorption and phosphorus excretion. In addition, there is a down regulation of vitamin D receptor and resistance to the actions of PTH at tissue level causing secondary hyperparathyroidism. [(1)]

Hyperparathyroidism plays a vital role in the excess morbidity and mortality in chronic kidney disease. As a result; patients are at increased risk of bone disease, extra osseous calcification, and death. [(1)] These changes probably begin early in the course of CKD, when Glomerular Filtration Rate (GFR) declines below 60 mL/min per 1.73 m^2. [(2)]

Cardiovascular disease accounts for 70% of all deaths in patients with CKD, with an overall mortality of 20% per year in patients on dialysis. [(3)] Left Ventricular Hypertrophy (LVH) is the most prevalent cardiac complication observed in CKD patients and is often associated with myocardial fibrosis, poor perfusion, and cell death. Hyperphosphatemia and hypercalcemia have been shown to promote calcification of the vasculature, myocardium, and cardiac valves. Vascular calcification, manifested in reduced vessel wall elasticity, increased intima media layer thickness is linked to LVH, and occurs with increased severity in dialysis patients versus non-CKD patients. [(4)] Vascular and soft-tissue calcifications are strong predicators of cardiovascular mortality among CKD patients.

Clinical care guidelines suggest that, at least annual testing and subsequent treatment of disorders of bone and mineral metabolism is essential early in the course of CKD when GFR is still 60 ml/min per 1.73 m^2. But various investigations suggest that less than 25% of patients reach the target levels for intact parathyroid hormone (PTH) measurement or control. [5]

AIM & OBJECTIVES

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REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

CHRONIC KIDNEY DISEASE:

Kidney - Anatomy

A kidney is a reddish brown, bean-shaped organ with a smooth surface. In an adult, it is about 12 centimetres long, 6 centimetres wide and 3 centimetres thick, and it is enclosed in a tough, fibrous capsule tunica fibrosa.

Location of the Kidneys

The kidneys lie on either side of the vertebral column, the upper and lower borders of the kidneys are generally at the levels of the twelfth thoracic and third lumbar vertebrae, respectively, although the positions of the kidneys may vary slightly with changes in posture and with breathing movements. The left kidney is usually about 1.5 to 2 centimetres higher than the right one.

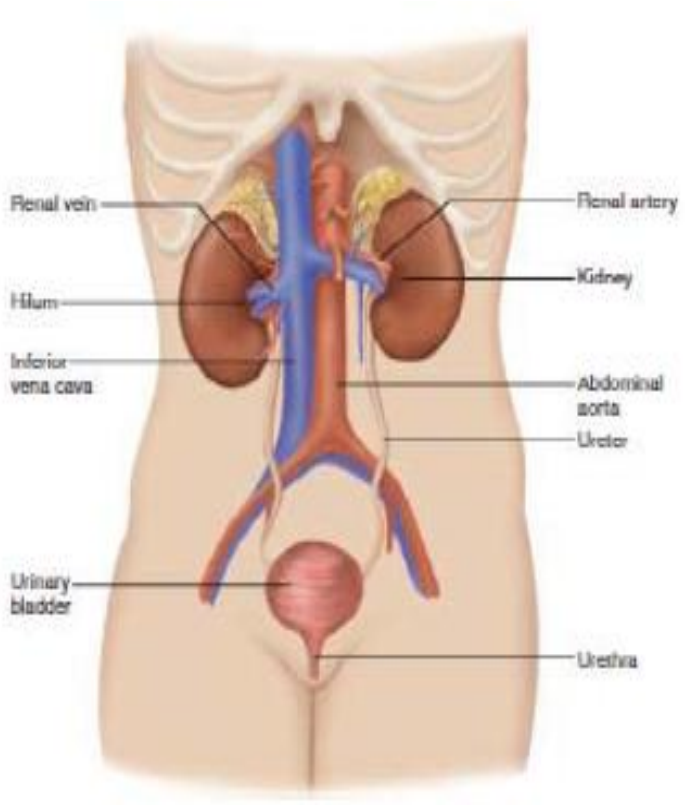

Fig 1: Location of kidney

Kidney Structure

The lateral surface of each kidney is convex, but its medial side is deeply concave. The resulting medial depression leads into a hollow chamber called the renal sinus. Through the entrance to this sinus pass blood vessels, nerves, lymphatic vessels and the ureter .The superior end of the ureter expands to form a funnel-shaped sac called the renal pelvis, which is located inside the renal sinus.

The pelvis is subdivided into two or three tubes called major calyces and they in turn are subdivided into eight to fourteen minor calyces. A series of small projections called renal papillae project into each minor calyx. The kidney includes two distinct regions: an inner medulla and an outer cortex. The renal medulla is composed of conical masses of tissue called renal pyramids, the bases of which are directed toward the convex surface of the kidney, and the apexes of which form the renal papillae. The tissue of the medulla appears striated because it consists of microscopic tubules that lead from the cortex to the renal papillae. The renal cortex dips into the medulla between the renal pyramids, forming renal columns and is surrounded by renal capsule.

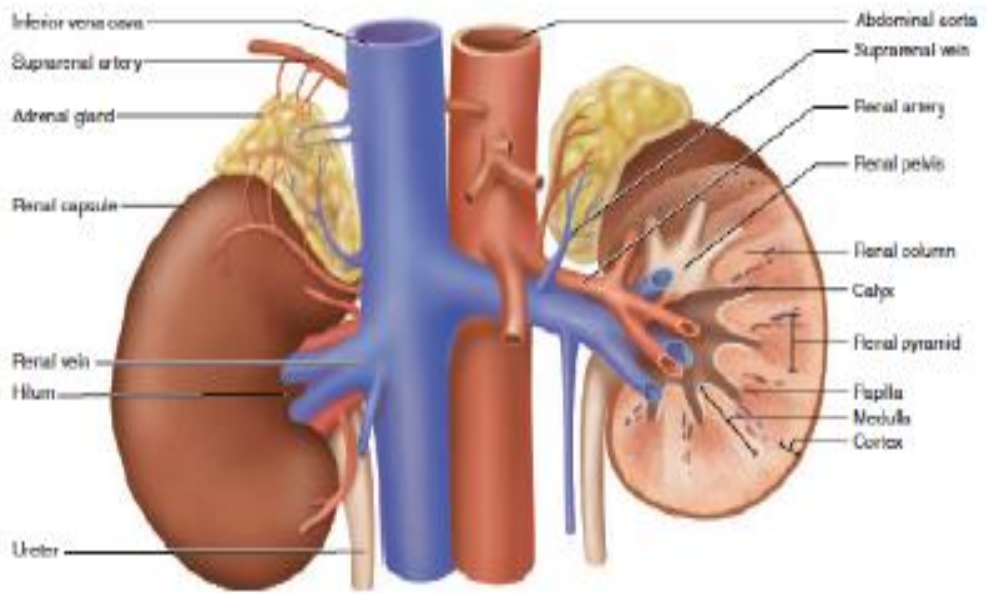

Fig 2: Structure of kidney

Renal Blood Vessels

The renal arteries, which arise from the abdominal aorta, supply blood to the kidneys. When a person is at rest, the renal arteries usually carry from 15% to 30% of the total cardiac output into the kidneys, although the kidneys account for only 1% of body weight. A renal artery enters a kidney through the hilum and gives off several branches, called the interlobar arteries, which pass between the renal pyramids. At the junction between the medulla and the cortex, the interlobar arteries branch to form a series of incomplete arches, the arcuate arteries which, in turn, give rise to interlobular arteries. The final branches of the interlobular arteries, called afferent arterioles lead to the nephrons, the functional units of the kidneys. Venous blood is returned through a series of vessels that generally correspond to the arterial pathways.

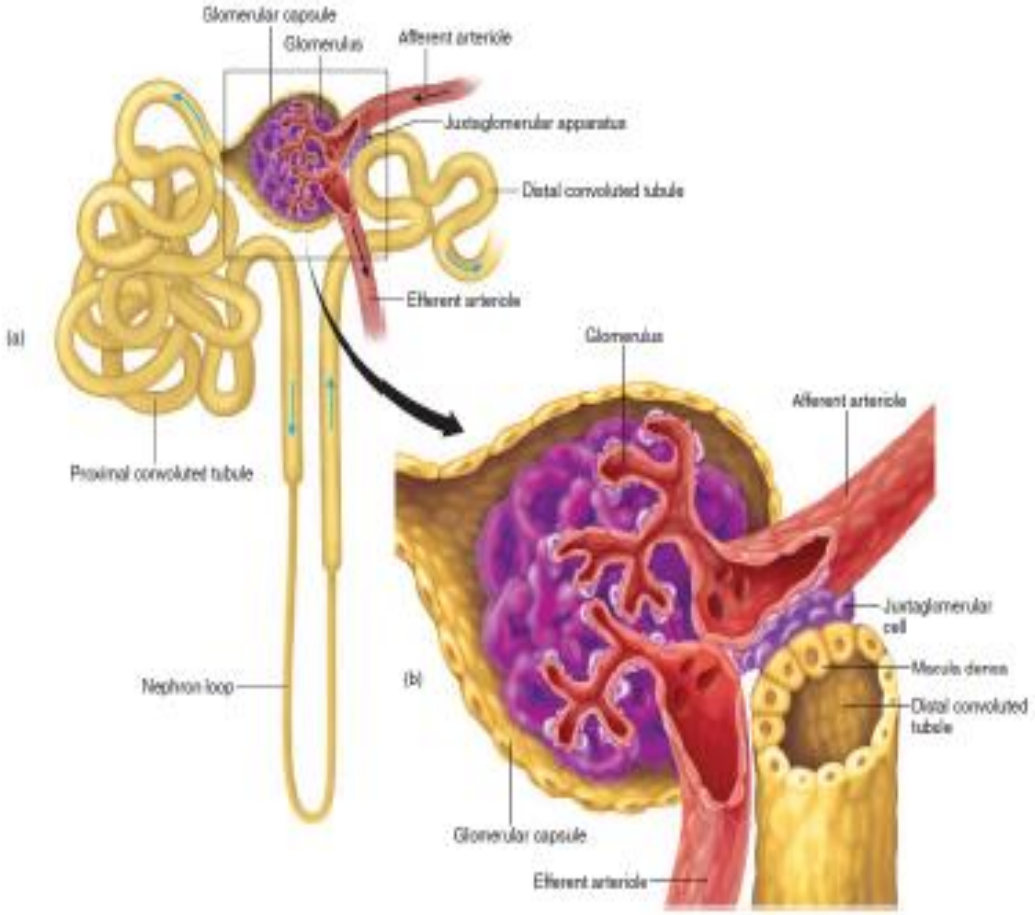

Fig 3: Structure of Nephron and Juxta Glomerular apparatus

Each kidney contains about one million functional units called nephrons, consists of a renal corpuscle and a renal tubule. A renal corpuscle consists of a filtering unit composed of a tangled cluster of blood capillaries called a glomerulus and a surrounding thin walled, sac like structure called a glomerular (Bowman′s) capsule. Afferent arterioles give rise to these capillaries, which lead to efferent arterioles. Filtration of fluid from the glomerular capillaries is the first step in urine formation. In the wall of the afferent arteriole near its attachment to the glomerulus, are large, vascular smooth muscle cells called juxtaglomerular cells. Together with the cells of the macula densa, they constitute the juxtaglomerular apparatus.

The glomerular filtration rate (GFR)

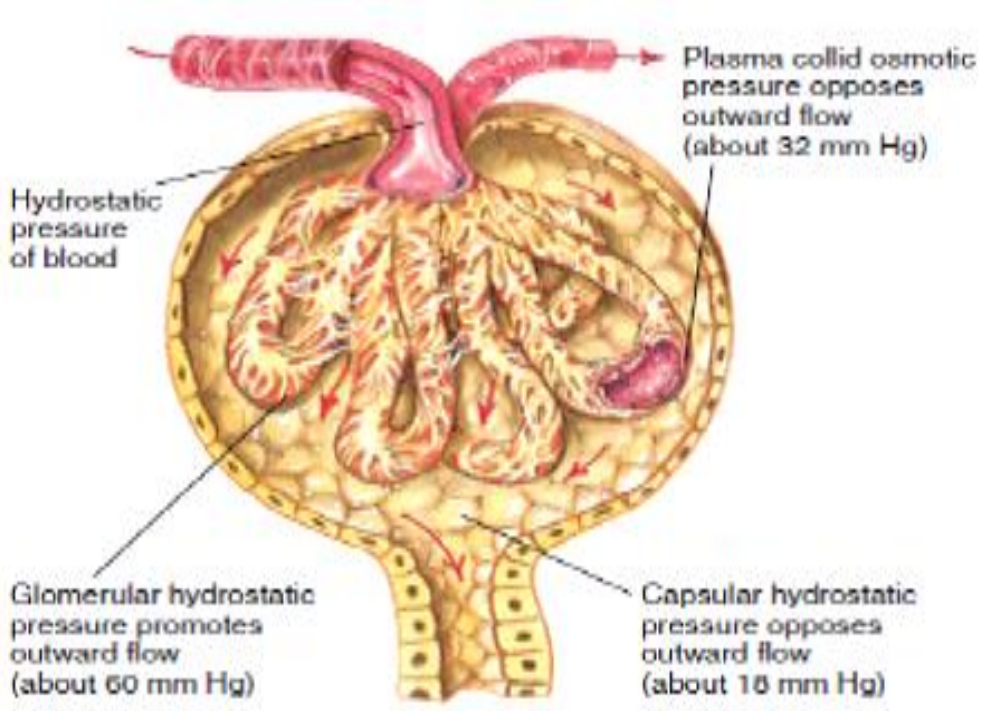

Fig 4: Factors effecting GFR

The glomerular filtration rate (GFR) is directly proportional to the net filtration pressure. Consequently, the factors that affect the glomerular hydrostatic pressure, glomerular plasma osmotic pressure, or hydrostatic pressure in the glomerular capsule will also affect the rate of filtration. The glomerular hydrostatic pressure is the most important factor determining net filtration pressure and GFR. Since each glomerular capillary is located between two arterioles, the afferent and efferent arterioles, any change in the diameters of these vessels is likely to change glomerular hydrostatic pressure, changing glomerular filtration rate. The afferent arteriole, through which the blood enters the glomerulus, may vasoconstrict in response to stimulation by sympathetic nerve impulses. If this occurs, net filtration pressure in that glomerulus decreases, and filtration rate drops. If on the other hand, the efferent arteriole through which the blood leaves the glomerulus vasoconstrict, blood backs up into the glomerulus, net filtration pressure increases.

The colloid osmotic pressure of the glomerular plasma also influences net filtration pressure and the rate of filtration. In other systemic capillaries, filtration occurs at the beginning of the capillary, but the osmotic effect of the plasma proteins predominates at the capillary and most filtered fluid is thus reabsorbed.

At rest, the kidneys receive approximately 25% of the cardiac output, and about 20% of the blood plasma is filtered as it flows through the glomerular capillaries. This means that in an average adult, the glomerular filtration rate for the nephrons of both kidneys is about 125 millilitres per minute. [6]

Functions of the kidney

The kidneys play a dominant role in regulating the composition and volume of the extracellular fluid (ECF). They normally maintain a stable internal environment by excreting in the urine appropriate amounts of many substances.

The kidneys perform a variety of important functions:

1. They regulate the osmotic pressure (osmolality) of the body fluids by excreting osmotically dilute or concentrated urine.
2. They regulate the concentrations of numerous ions in blood plasma, including sodium, potassium, calcium, magnesium, chloride, bicarbonate, phosphate and sulfate.
3. They play an essential role in acid–base balance by excreting H^+ when there is excess acid or $HCO3^-$ when there is excess base.
4. They regulate the volume of the ECF by controlling sodium and water excretion.
5. They help regulate arterial blood pressure by adjusting sodium excretion and producing various substances (e.g., Renin) that can affect blood pressure.
6. They eliminate the waste products of metabolism including urea, the main nitrogen containing end product of protein metabolism in humans, uric acid an end product of purine metabolism, and creatinine an end product of muscle metabolism.
7. They remove many drugs (e.g., Penicillin) and foreign or toxic compounds.
8. They are the major sites of production of certain hormones, including erythropoietin and 1, 25-dihydroxy vitamin D3.
9. They degrade several polypeptide hormones, including insulin, glucagon, and parathyroid hormone.
10. They synthesize ammonia, which plays a role in acid–base balance. [6]

CHRONIC KIDNEY DISEASE

Chronic kidney disease is a worldwide public health problem. It is the ninth leading cause of death. A trend towards increased incidence and prevalence is being reported worldwide with epidemic proportions in many countries.

EPIDEMIOLOGY

In United States, there is a rising incidence and prevalence of kidney failure with poor outcomes and high cost. The incidence of CKD is expected to rise annually by 5-8%. [7] The number of patients treated with dialysis or transplantation is projected to increase from almost 5, 00, 000 people in 2005 to 8, 00, 000 by 2020. A similar tendency is observed in Europe and United Kingdom. The estimated prevalence rates of chronic kidney disease in India are 800 and end-stage renal disease 200 per million inhabitants, respectively. [8] In South India, the main causes of CKD in decreasing order of prevalence are diabetic nephropathy (29.6%), chronic interstitial nephritis (20.4%), chronic glomerulonephritis (17.4%), and hypertensive nephropathy (11%). [9] Unfortunately chronic kidney disease is "under diagnosed" and "under-treated" resulting in lost opportunities for prevention. [10]

The Kidney Disease Outcomes Quality Initiative (KDOQI) of the National Kidney Foundation (NKF) defines "Chronic Kidney Disease as either kidney damage or a glomerular filtration rate (GFR) of less than 60 mL/min/1.73 m^2 for 3 or more months". [11] In 2002; KDOQI published its classification of the stages of chronic kidney disease, as follows:

Stage 1: Kidney damage with normal or increased GFR (>90 mL/min/1.73 m^2)

Stage 2: Mild reduction in GFR (60-89 mL/min/1.73 m^2)

Stage 3: Moderate reduction in GFR (30-59 mL/min/1.73 m^2)

Stage 4: Severe reduction in GFR (15-29 mL/min/1.73 m^2)

Stage 5: Kidney failure (GFR < 15 mL/min/1.73 m^2 or on dialysis).

The KDOQI definition and classification of chronic kidney disease allow better communication among physicians and facilitate intervention at the different stages. Patients with chronic kidney disease stages 1-2 are generally asymptomatic; clinical manifestations typically appear in stages 3-5.

Risk Factors for CKD

According to KDOQI guidelines, the risk factors for the development of CKD can be divided into following**:**

Susceptibility factors: There is increased susceptibility to kidney damage seen in older age, family history of chronic kidney disease, reduction in kidney mass and low birth weight.

Initiation factors: They directly initiate kidney damage like Diabetes, high blood pressure, autoimmune diseases, systemic infections, urinary tract infections, urinary stones, lower urinary tract obstruction and drug toxicity.

Progression factors: They cause faster decline in kidney function after initiation of kidney damage they include higher level of proteinuria, higher blood pressure, poor glycaemic control in diabetes, smoking.

End-stage factors: These factors increase morbidity and mortality patients they include lower dialysis dose (Kt/V), temporary vascular access, anaemia, low serum albumin level and late referral. [(12)]

PATHOPHYSIOLOGY OF CKD

Approximately 1 million nephrons are present in each kidney, contributing to the total GFR. In the case of renal injury, the kidney has an ability to maintain GFR, by methods of hyperfiltration and compensatory hypertrophy of the remaining healthy nephrons. This, nephrons adaptability allows for continued normal clearance of plasma solutes. Plasma levels of substances such as urea and creatinine start to show significant increases only after total

GFR has decreased to 50%. A rise in plasma creatinine from a baseline value of 0.6 mg/dL to 1.2 mg/dL in a patient even though still within the reference range actually represents a loss of 50% of functioning nephron mass.

Decreased renal function interferes with the kidneys ability to maintain fluid and electrolyte homeostasis. The ability to concentrate urine declines early and is followed by decrease in ability to excrete phosphate, acid, and potassium. When renal failure is advanced (GFR $\leq$ 10 mL/min/1.73 m^2) the ability to dilute urine is lost; thus urine osmolality is usually fixed close to that of plasma (300 to 320 mOsm/kg) and urinary volume does not respond readily to variations in water intake. Plasma concentrations of creatinine and urea which are highly dependent on glomerular filtration begin a nonlinear rise as GFR diminishes. [13]

Abnormalities of calcium, phosphate, parathyroid hormone (PTH), vitamin D metabolism and renal osteodystrophy can occur. Decreased renal production of calcitriol contributes to hypocalcemia. Decreased renal excretion of phosphate results in hyperphosphatemia. Secondary hyperparathyroidism is common and can develop in renal failure before abnormalities in calcium or phosphate concentrations occur. For this reason monitoring PTH levels in patients with moderate CKD is recommended even before hyperphosphatemia occurs.

CKD - Clinical Features

Lethargy, fatigue, anorexia, decreased mental acuity are commonly the earliest manifestations of renal failure. With more severe renal insufficiency neuromuscular symptoms may be present including coarse muscular twitches, peripheral sensory and motor neuropathies, muscle cramps, hyper reflexia, and seizures. Anorexia, nausea, vomiting,

weight loss, stomatitis, unpleasant taste, discoloration of skin may be seen. Pruritus and under nutrition, leading to generalized tissue wasting are prominent features of CKD.

Hypertension is present in more than 80% of patients with advanced CKD, usually related to hypervolemia, and occasionally due to activation of the Renin-Angiotensin-Aldosterone System (RAAS). Heart failure caused by hypertension or coronary artery disease and renal retention of Na and water may lead to dependent edema. [13]

Diagnosis

Kidney damage is usually ascertained by markers rather than by kidney biopsy. According to the KDOQI, persistent proteinuria is the principal marker of kidney damage. [14] An elevated albumin– creatinine ratio in urine samples is usually considered abnormal. [15] [16] But, estimation of level of Glomerular filtration rate is the best measure of overall kidney functions both in health and disease. [17] The normal level of GFR varies according to age, sex and body mass. Normal GFR in young adults is approximately 120 to 130 mL/min per 1.73 m^2 and declines with age. [18] A GFR level less than 60 mL/min per 1.73 m^2 represents loss of half or more of the adult level of normal kidney function. Below this level, the prevalence of complications of chronic kidney disease increases. Other markers of kidney damage include urinary sediments like WBCs and RBCs, abnormalities in electrolytes and urine osmolality, and abnormal findings on imaging studies.

Estimation of GFR

GFR was routinely measured using Creatinine clearance, but now various formulas have been developed for calculation of GFR, taking into account age, sex, race and body mass. The most commonly used equations are Cockcroft-Gault formula, the Modification of Diet in Renal Disease (MDRD) formula and CKD-EPI creatinine equation.

Cockcroft–Gault equation [19]:

$$C\ Cr = \frac{(140 - Age) \times Weight}{72 \times S\ Cr} \times 0.85 \text{ (if female)}$$

MDRD study equation [20]:

GFR (mL/min per 1.73 m2) = $186 \times (S\ Cr)^{-1.154} \times (age)^{-0.203} \times 0.742$ (if female) × 1.210 (if African-American)

CKD-EPI equation [21]:

$GFR = 141 \times \min(S\ Cr/\kappa, 1)^{\alpha} \times \max(S\ Cr/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] × 1.159 [if black]

Here *C Cr* is creatinine clearance, *S Cr* is serum creatinine concentration in mg/dL, age is in years, and weight is in kg. κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of S Cr/κ or 1, and max indicates the maximum of S Cr/κ or 1

The MDRD study equation has many advantages. It is more accurate and precise than the Cockcroft–Gault equation for persons with a GFR less than approximately 90 mL/min per 1.73 m^2. The CKD-EPI creatinine equation is based on the same four variables as the MDRD Study equation, but uses a 2-slope spline to model the relationship between estimated GFR and serum creatinine, and a different relationship for age, sex and race. The equation was reported to perform better and with less bias than the MDRD Study equation, especially in patients with higher GFR. This results in reduced misclassification of CKD. The CKD-EPI creatinine equation is used in our study to estimate GFR.

Complications of CKD

1. Anaemia and coagulation disorders
2. Bone disorders and osteoporosis
3. Acidosis
4. Cardiovascular complications
5. Progression to end stage renal disease.

Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD)

Definition of Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD): A systematic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following:

1. Abnormalities of PTH, calcium, phosphorus, or vitamin D metabolism
2. Abnormalities of bone turnover, mineralization, volume, linear growth or strength
3. Vascular or other soft tissue calcification. [22]

Mineral disturbances are common complications of CKD, beginning early in the course of disease. In stage 3 CKD, the kidney neither is able to fully excrete phosphorus load nor can convert vitamin D into its active metabolite calcitriol. This leads to a compensatory secondary hyperparathyroidism. [23] Elevated PTH and decreased calcitriol levels are found in 40% of patients with stage 3 and 80% of patients with stage 4. [24] These minerals and endocrine functions disrupted in CKD are critically important in the regulation of bone remodelling. As a result, bone abnormalities like altered remodelling and loss of bone volume are found in almost all patients with CKD. [25] The skeletal changes result in an increased prevalence of hip fracture compared to the general population across the entire range of CKD stages 3-5 patients. [26]

Derangements in mineral metabolism are also associated with cardiovascular disease and all-cause mortality. [27] [28] [29] Cardiovascular disease accounts for 70% of all deaths in patients with CKD, with an overall mortality of 20% per year in patients on dialysis. In

individuals with kidney failure on dialysis, cardiovascular mortality rates are 10 to 500 times higher than in the general population. [30] Individuals at earlier stages of CKD not yet on dialysis (stages 3-4) have 17-times more chances to die of cardiovascular disease rather than progressing to dialysis. [31]

Multiple cross-sectional studies in dialysis patients have found disordered mineral metabolism, including hyperphosphatemia and hyperparathyroidism, increasing the risk of cardiovascular and all-cause mortality. [32] [33] One mechanism by which abnormal mineral metabolism may increase cardiovascular risk is by inducing or accelerating arterial and valvular calcification. Patients on dialysis have 2 to 5 fold more coronary artery calcification than age-matched individuals with angiographically-proven coronary artery disease. [34]

In patients not yet on dialysis, there is also increased coronary artery calcification compared to matched controls. [35] Peripheral artery calcification can lead to claudication and systolic hypertension; this in turn can leads to increased cardiac after load resistance and left ventricular hypertrophy. Coronary artery calcification can lead to cardiac ischemia and sudden death and this is the leading cause of cardiovascular death in patients on dialysis. [36] In patients with CKD, there is emerging evidence to support an interrelationship between bone, vascular disease and disordered mineral metabolism.

These changes led to an initiative in 2006 by KDIGO to clarify and update nomenclature. As a result, the new term "Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD)" was put forth to describe a clinical syndrome composed of mineral metabolism abnormalities, renal osteodystrophy and extra-osseous calcification including vascular calcification.

A major component of CKD-MBD is vascular calcification, which is highly prevalent in CKD patients. Calcification can occur in intimal plaques and atheroma and also in the medial layer, especially in elastic containing arteries. [37] [38] In patients starting on dialysis, 60-70% of patients have significant coronary artery calcification by CT-based imaging. [39]

Pathologically on autopsy studies, the medial layer of coronary arteries is thicker in CKD patients than in controls, and medial coronary calcification is found in 20% of patients with CKD stages 4 and 5. [37]

Calcification is also very common in the peripheral arteries; with demonstrating both intimal and medial calcification. [40] Both coronary artery and peripheral artery calcification are associated with increased mortality in patients on dialysis. The risk factors associated with arterial calcification include advanced age, diabetes, obesity, hypertension, dyslipidemia, inflammatory markers, hypoalbuminemia, use of calcium containing phosphate binders and disordered mineral metabolism. [41] Vascular calcification was previously thought to be a passive process, due to the elevations in calcium and phosphorus observed in patients with advanced CKD. Recent evidence confirms this is an active process due to a series of events. The initial step is thought to be a transformation or de-differentiation of vascular smooth muscle cells (VSMCs) to osteogenic or chondrogenic-like cells. These cells can then form matrix vesicles or apoptotic bodies that mineralize on an extracellular matrix, presumably in a manner similar to bone.

The existence of abnormal bone remodelling in CKD may accelerate the process by providing excess calcium and phosphate for the matrix vesicles. The overall pathogenesis is regulated by a balance of pro-calcifying factors and inhibitors. Unfortunately in CKD, the procalcifying factors including hyperphosphatemia and hyperparathyroidism are common, and inhibitors such as fetuin-A and matrix GLA protein are reduced.

PARATHYROID HORMONE

Parathyroid Gland Anatomy

The parathyroid glands were first described in 1850. Thirty years later, an illustrated anatomical and histological description in animals and humans was given by Ivar Sandstrom, a medical student from Uppsala. [42] In the 1980s, PTH was sequenced, its gene and its receptor were cloned and improved chemiluminescent immunoassays for intact PTH were developed.

Structure of the Glands

The parathyroid glands are small, yellowish-brown, ovoid or lentiform structures, lying between the posterior lobar borders of the thyroid gland and its capsule. They are commonly 6 mm long, 3-4 mm across, and 1-2 mm from back to front, each weighing about 50mg, two on each side, superior and inferior. The parathyroid glands have a rich blood supply from the inferior thyroid arteries or from anastomoses between the superior and inferior vessels. [6] Parathyroid activity is controlled by variations in blood calcium level; it is inhibited by a rise and stimulated by a fall. [6]

Biochemistry

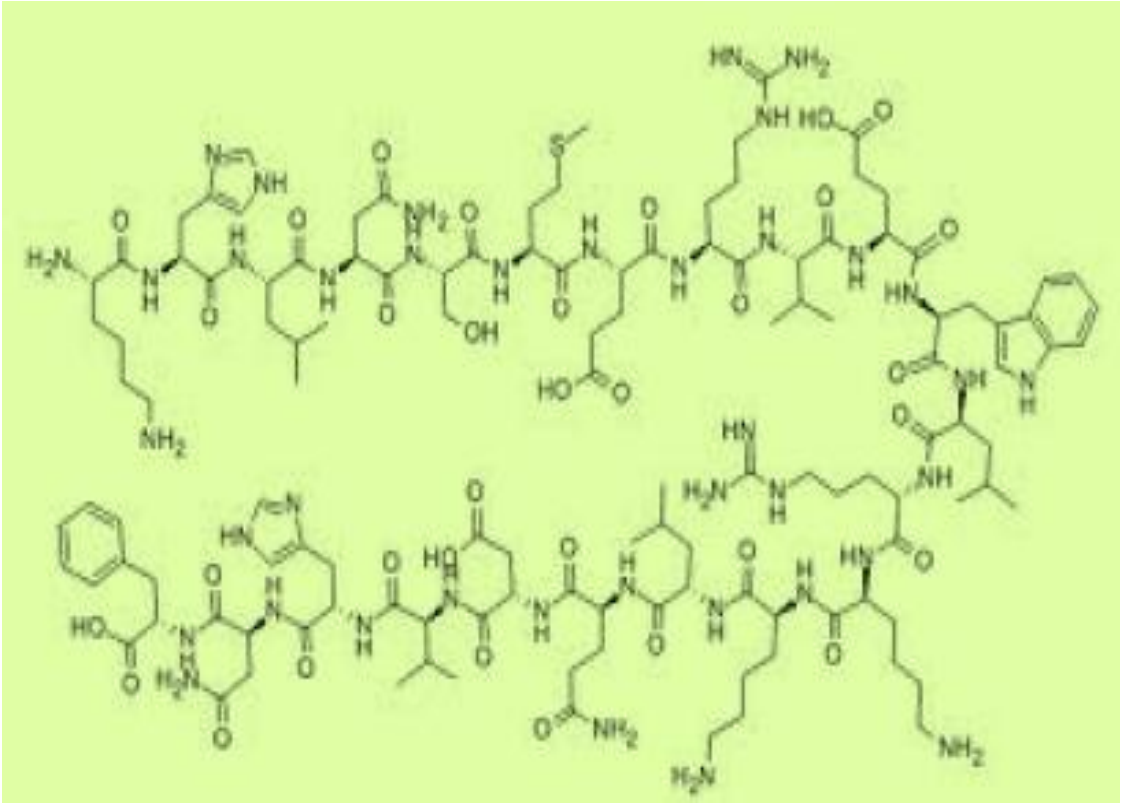

Fig 5: Structure of Parathyroid Hormone.

PTH is an 84 amino acid peptide with a molecular weight of 9500 Da and a short half-life of 2–3 min in the circulation. PTH binds to specific receptors on the membrane of target cells: renal & bone cells, fibroblasts, chondrocytes, vascular smooth muscle, adipocytes and placental trophoblasts. [43]

PTH synthesis and degradation

PTH is synthesized as a 115-amino acid polypeptide called pre-pro-PTH, which is cleaved within parathyroid cells at the N-terminal portion first to pro-PTH (90 amino acids) and then to PTH (84 amino acids). The latter is the major storage, secreted and biologically active form of the hormone. [44] Only the (1–34) amino acids fragments retain biological activity. The biosynthetic process is estimated to take less than one hour. The N-terminal cleaved pre-sequence is rich in hydrophobic amino acids that are necessary for transport into

the endoplasmic reticulum, while the basic pro-peptide directs accurate cleavage of pro-PTH into the mature 1-84 molecule. The C-terminal portion of PTH is also essential for the PTH secretory process. PTH (1-84) is secreted by exocytosis within seconds after induction of hypocalcemia. [43] Calcium regulates not only the release but also the synthesis and degradation of PTH. Once secreted, PTH is rapidly cleared from plasma through uptake principally by the liver and kidney, where PTH 1-84 is cleaved into amino and carboxyl terminal fragments, which are then cleared by the kidney. [45] In CKD, amino-terminal fragments remain active and carboxy-terminal fragments accumulate.

Mechanism of action of parathyroid hormone

Physiological Role:

The parathyroid glands are responsible for secreting PTH which maintains calcium homeostasis. PTH is a polypeptide hormone. Fluctuations in serum calcium concentration are detected by calcium receptors (CaR) which are G-protein coupled receptors located on the chief cells of the parathyroid gland that mediate the secretion of PTH. [46] Stimulated by low serum calcium levels, PTH secretion increases and up-regulates the expression of 1α-hydroxylase in the kidney. This enzyme is responsible for the production of vitamin D or calcitriol. [47]

Under hypocalcemic conditions, PTH stimulates the synthesis of calcitriol, correcting the calcium imbalance by

1. Increasing absorption of calcium from the gastrointestinal tract
2. Conserving calcium that would ordinarily be excreted by the kidneys
3. Releasing calcium from bone. [48]

Calcitriol and PTH are both capable of directly regulating serum mineral levels.

The effects of PTH on the intestine are accomplished indirectly through the actions of calcitriol. Calcitriol has been shown to up-regulate the expression of calcium channel and transporter proteins, correcting hypocalcemia at the transcriptional level thus increases calcium absorption. [49]

The effects of elevated PTH levels on the skeleton include the stimulation of calcium mobilization from bone and the direct regulation of osteoblasts apoptosis to correct hypocalcemia. [50] Completing the feedback loop**,** both elevated calcium and calcitriol levels can suppress PTH secretion but via different mechanisms. Sela-Brown et al have shown that calcitriol suppresses PTH synthesis at the transcriptional level whereas calcium regulation is post transcriptional. [51] Abnormalities in mineral homeostasis usually originate from the failure to maintain this critical feedback loop, often leading to hyperparathyroidism.

PTH is secreted by the parathyroid glands in response to hypocalcemia, hyperphosphatemia, and calcitriol deficiency. Minute-to-minute concentrations of PTH are most sensitive to low ionized calcium concentrations. The sensitivity of this response may be blunted in the presence of hyperphosphatemia in CKD.

NORMAL FUNCTIONS OF PTH

Effects of PTH on the kidney

The distribution of PTH receptors and that of Ca^{2+} receptors overlap in the distal nephron, allowing calcium to have a direct effect on Ca^{2+} receptors and an indirect influence through modulation of plasma PTH concentrations on the renal component of calcium homeostasis. The intracellular mediator for PTH effects is intracellular cyclic adenosine monophosphate (cAMP), whose urinary secretion is a biochemical marker of PTH activity.

The effects of PTH on the kidney include:

(i) The major physiological effect of PTH is enhancement of Ca^{2+} reabsorption. This is due to effects on: the thick ascending loop of Henle it increases the transepithelial voltage gradient that drives passive Ca^{2+} transport.

(ii) Increased phosphate excretion. PTH acts on the proximal and distal convoluted tubules and inhibits Na^{+} - dependent phosphate transport.

(iii) Increased bicarbonate clearance and alkalinisation of the urine result from inhibition of bicarbonate reabsorption in the proximal renal tubule. In patients with primary hyperparathyroidism, excessive secretion of PTH leads to a type of renal tubular acidosis.

(iv) Inhibition of Na^{+} reabsorption in the proximal tubule leads to an increase in Na^{+} loading of the distal tubule. Na^{+} is reabsorbed proportionally more than the associated water, leading to an increase in free water clearance.

(v) Increased activity of vitamin D1α hydroxylase.

(vi) In primary hyperparathyroidism, the renal effects of PTH are observed as hypercalciuria, hypophosphataemia, hyperchloremic acidosis, polyuria, polydypsia and an increased excretion of nephrogenous fraction of cAMP.

Effects of PTH on the bone

PTH produces both anabolic and catabolic effects which can be distinguished as early phase which includes mobilization of Ca^{2+} from bone in rapid equilibrium with the extracellular fluids and late phase increased by promoting reabsorption and bone remodelling. Osteoblasts are probably the primary bone cells that interact directly with PTH while osteoclasts seem to be devoid of PTH receptors. PTH inhibits osteoblasts and stimulates osteoclast mediated bone resorption, leading to an increase in alkaline phosphatase and

increased urinary hydroxyproline. In primary hyperparathyroidism, changes in alkaline phosphatase and hydroxyproline are markers of bone disease.

Effects of PTH on the intestine

PTH does not directly affect gastrointestinal absorption of $Ca^{2+.}$ Its effects are mediated indirectly through regulation of synthesis of 1, 25(OH) $_2$ D_3 in the kidney.

Extracellular calcium

Extracellular calcium is the major determinant of the rate of PTH secretion; slight reductions in calcium levels increase promptly the rate of PTH secretion. The initial changes in rate of PTH secretion in response to low calcium take place within seconds because of the release of preformed hormones from storage granules. Within 15–30 min, there is also an increase in the net rate of PTH synthesis. If the hypocalcaemic stimulus persists, modest increases in the amount of PTH mRNA take place early. Prolonged hypocalcaemia promotes parathyroid cellular hypertrophy and proliferation within days to weeks. The human Ca^{2+} receptor is encoded by a gene on chromosome 3q13-21 and consists of 1078 amino acids. (52)

Calcium

Serum calcium levels are controlled tightly in the range of 8.5-10.5 mg/dL. Total body stores are 1,000g among this 99% in bone, 0.9% intracellular, and 0.1% extracellular. Extracellular calcium is measured as total calcium: In this 50% is free, 10% is bound to anions and 40% is bound to albumin. Average dietary intake of calcium is 500- 1000mg/day. Calcium absorption occurs across intestinal epithelium through vitamin D-dependent transporters. Bioavailability of calcium from foods is altered by phytate and oxalate. Absorbed calcium enters into 3 compartments: Blood, soft tissue and bone. (53)

Renal Handling of Calcium

Reabsorption: 60-70% is reabsorbed passively in proximal tubules with sodium and water reabsorption. 10% is reabsorbed in the thick ascending limb. The rest is reabsorbed through transcellular pathways in the distal convoluted tubule, connecting tubule and cortical collecting duct.

Calcium-Sensing Receptor (CaSR)

It is a G-protein coupled protein that binds calcium and identifies even slight changes in ionized calcium levels; decreased ionized calcium stimulates PTH secretion. CaSR is expressed in parathyroid cells, thyroid C cells, intestine, kidney, and bone. In kidneys, CaSR is in mesangial cells and throughout tubules. Activation of CaSR on the thick ascending limb decreases paracellular calcium reabsorption. [53]

Changes in concentrations of calcium is sensed by chief cells through a cell-surface, seven-transmembrane, G protein–coupled receptor, the CaSR [54] and receptor activity results in rapid alterations in PTH secretion. [55] After the induction of abrupt and sustained hypocalcemia, plasma concentrations of PTH increase within 1 minute, peak at 4 to 10 minutes, and thereafter decline gradually to approximately 60% of the maximum at 60 minutes, despite ongoing and constant hypocalcemia. Abrupt restoration of normocalcemia from the hypocalcemic state causes levels of PTH to decrease with an apparent half-life of approximately 3 minutes. In addition to its role in the parathyroid gland, the CaSR plays an important role in regulating calcium reabsorption in the thick ascending limb of the loop of Henle. [56]

Phosphorus

Serum phosphorus concentration ranges from 2.5-4.5 mg/dL; total-body stores of phosphorus equal 700 g. Of total-body stores, 85% is in bone as hydroxyapatite; 14% intracellular; and 1% extracellular.

Diet contains about 1000-1400 mg/day of phosphorus; two-thirds excreted in urine, one third excreted in stool. Processed foods and foods rich in animal-based protein are high in Phosphorus and thus it is difficult for patients with CKD to control serum phosphorus levels by diet alone. 60-70% of dietary phosphorus is absorbed in all intestinal segments. [53]

Renal Handling of Phosphorus

Inorganic phosphorus is filtered by glomeruli, and then 70-80% is reabsorbed in proximal tubule through the Sodium-Phosphate co-transporter 2b. 20-30% of filtered phosphorus is reabsorbed in distal tubule. Renal phosphorus excretion is sensitive to serum phosphorus levels. PTH and Fibroblast Growth Factor 23 (FGF-23) increase phosphorus excretion. Phosphorus depletion decreases its own excretion. [53]

Fibroblast Growth Factor 23

It belongs to a group of molecules called phosphatonins. Phosphatonins are hormones that regulate phosphorus excretion. Three phosphatonins have been identified FGF-23 is one among them. They are produced almost exclusively in osteocytes and bone-lining cells, also found in heart, liver, thyroid, parathyroid, intestine and skeletal muscle.

FGF-23 receptor on the proximal tubule requires a co-receptor (Klotho) for signal transduction. Klotho is found in the distal renal tubule and parathyroid gland. They are down-regulated in aging and CKD. FGF-23 has the following actions down-regulates luminal sodium/phosphate co transporters in the proximal tubule, decreasing phosphorus reabsorption and therefore increasing its excretion. It Inhibits 1-hydroxylase, decreasing the conversion of

25-hydroxyvitamin D to 1, 25-Dihydroxy vitamin D (calcitriol), it also inhibits PTH secretion. FGF-23 gene expression in bone is stimulated by elevated phosphorus, PTH and calcitriol levels. Both FGF-23 and PTH lead to increased phosphorus excretion. Hypocalcemia stimulates PTH and therefore in low calcium high-phosphorus states the action of PTH predominates where as in high-calcium high-phosphorus states, the action of FGF-23 predominates. [53]

Parathyroid hormone in Chronic Kidney Disease

Pathophysiology

PTH is associated significantly with mortality in observational studies at levels varying from 400-600 pg/mL, depending on the population analyzed. There are inconsistent data for the underlying bone histology by biopsy in dialysis patients for whom PTH levels were maintained in the range of 150-300 pg/mL recommended by KDOQI guidelines. Given these issues, recent KDIGO guidelines recommend extremes of risk for PTH at less than 2 times the lower-limit and greater than 9 times the upper-limit values of the specific assay used. However, trends of PTH levels within that range should be evaluated and medications should be adjusted as needed. [53]

Secondary hyperparathyroidism (SHPT)

Secondary hyperparathyroidism describes a complex alteration in bone and mineral metabolism that occurs as a direct result of Chronic Kidney Disease (CKD). Patients with mild CKD may be asymptomatic and therefore may not be identified until the pathology of SHPT has begun. Identifying patients at risk and evaluating for SHPT is imperative because early intervention may slow or arrest the progression of both bone and cardiac disease.

A common complication manifested in elevated parathyroid hormone (PTH) levels as a direct result of decreased renal function, vitamin D deficiency, and impaired mineral

metabolism. [57] SHPT arises in most patients during the progression of CKD and is associated with several co morbidities, including renal osteodystrophy (ROD), extra skeletal calcification, and cardiovascular disease (CVD), resulting in increased mortality. [58]

Several treatment options are available to slow the progression of CKD. A component of CKD care often overlooked and undertreated by both the primary care physician and the nephrologist is the management of SHPT, which can lead to CVD and ROD if untreated. Since kidney disease is often diagnosed comparatively late in many patients, the staging at diagnosis is often stage 3 or 4, the same time that these extra renal complications begin to appear. [59] [60] [61] Although hyperphosphatemia appears to be particularly important in the development of SHPT, the complication often occurs early in stage 3 of kidney failure, before the development of hyperphosphatemia. [62] [63]

Hyperphosphatemia and an elevated Calcium X Phosphorous product, other widespread observational findings in CKD patients are considered to be independent risk factors for CVD, including hypertension, Left Ventricular Hypertrophy (LVH), increased serum creatinine levels, and microalbuminuria. [64] Traditional risk factors such as smoking, diabetes, hypertension, dyslipidemia, and obesity can be present at all stages of CKD and may also increase the risk of CVD. [65]

Renal Osteodystrophy

The processes of bone absorption and resorption are closely regulated in healthy individuals. ROD arises as a consequence of bone remodelling dysregulation. While the pathogenesis of ESRD is similar across patients, the pathogenesis of ROD varies from high- to low-bone turnover. Elevated levels of PTH stimulate bone demineralization and lead to high-bone turnover, a condition characterized by accelerated rates of bone absorption and resorption. The new bone is structurally inferior and fragile and carries an increased risk of

fractures. The classic histological form of ROD is osteitis fibrosa, which arises from increased bone remodelling and fibrosis of the marrow. [66]

Cardiovascular Disease

The soft-tissue and vascular calcification that accompanies SHPT in advanced CKD is associated with a high risk of cardiac events. LVH is the most prevalent cardiac complication observed in CKD patients and is often associated with myocardial fibrosis, poor perfusion, and cell death. [67] [68] In a cross sectional study, Saleh et al found PTH to be an independent predictor of LVH among patients in the upper PTH percentiles. [69] Nasri et al. analyzed the influence of PTH on myocardial function. In their cross sectional study in hemodialysis patients, they determined that excess PTH played a significant role in the development of LVH and reduced left ventricular ejection fraction. [70]

The relationship between elevated PTH and LVH was further explored in a retrospective study by Goto et al., who determined that parathyroidectomy in CKD patients with advanced SHPT led to a significant improvement of left ventricular ejection fraction and function. [71] A prospective study by Park et al. confirmed the association with SHPT, finding that PTH-suppressive calcitriol therapy led to a regression in myocardial hypertrophy in dialysis patients. [72] Hyperphosphatemia and hypercalcemia have been shown to promote calcification of the vasculature, myocardium, and cardiac valves. [73] [74]

Vascular calcification, manifested in reduced vessel wall elasticity, increased intima media layer thickness and enhanced pulse-wave velocity, has been linked to LVH, and occurs with increased severity in dialysis patients versus non-CKD patients. [75] Vascular and soft-tissue calcifications are strong predictors of cardiovascular mortality among CKD patients. [76]

The exact mechanism of calcification is not known but it is proposed that as the calcium, a divalent cation, and phosphorus, a monovalent anion, have a high binding affinity

for each another. In serum, as the concentration of one or both ions increases, there is an increased risk for an ionic bond to form, creating an insoluble complex. This process may lead to extra skeletal calcification and potentially calciphylaxis or cardiac disease. [77] Additionally, the precipitation may decrease serum calcium concentrations, further stimulating PTH secretion. In fact, PTH production and secretion may be stimulated by hypocalcemia, hyperphosphatemia, and vitamin D deficiency. [78] Because PTH is chiefly responsible for preventing hypocalcemia, it stimulates osteoclasts to lyse bone, releasing calcium into the serum.

Retrospective studies by Block et al and Ganesh et al suggest that hyperphosphatemia (> 6.5 mg/dL) and increased Calcium x Phosphorus product (>72 mg/dL) are factors that contribute to the high mortality rate in dialysis patients. [79] [80]

CKD and Phosphorus

Phosphorus homeostasis is impaired when the GFR is 60 mL/min as GFR decreases there is a gradual increase in serum phosphorus levels. During this period, normal phosphorus levels are maintained by continuous increases in FGF-23 and PTH levels. Eventually, this compensatory mechanism is overwhelmed when GFR decreases to 30 mL/min, and serum phosphorus levels may increase to higher than the reference range. Hyperphosphatemia also leads to inhibition of calcitriol synthesis, which stimulates further PTH production; together these processes trigger the development of secondary hyperparathyroidism in CKD.

An observational data suggest that hyperphosphatemia is connected to increased morbidity and mortality in CKD. Various studies of patients with CKD stage 5, the phosphorus level associated with increased mortality varies from > 5.5 to > 7 mg/dL. Even in the non-CKD population, serum phosphorus level in the high-normal ranges is associated with increased risk of cardiovascular and all cause mortality. No interventional study has

shown that decreasing phosphorus to a certain target level is associated with better outcomes. [53]

Calcium Abnormalities in CKD

In CKD stages 2-3, serum calcium levels are maintained in the reference range at the cost of secondary elevations in PTH levels. Intestinal calcium absorption is impaired in CKD due to decreased calcitriol levels, but still proportional to calcium intake. Urinary calcium excretion decreases as CKD progresses due to PTH-associated increased reabsorption and decreased filtered fraction of calcium. In CKD, intestinal absorption is not equal to urinary excretion. The ability of bone to take up calcium depends on bone turnover. Patients with lower bone turnover (adynamic bone) are less able to take up calcium. When tubular excretion of calcium is decreased, these patients have a net positive calcium balance. Given a net positive calcium balance in late CKD, KDOQI guidelines recommend maximum total elemental calcium intake of 2 g/day. [53]

Calcium Phosphorus product:

Hyperphosphatemia is a common problem in patients with CKD has been associated with the progression of secondary hyperparathyroidism, deposition of calcium in soft tissues, and vascular calcification. [81] Higher serum PO4, Calcium X Phosphorus product is associated with increased coronary artery calcification. In addition to this, it also contributes to vascular smooth muscle cell proliferation and compromise flow in the coronary microcirculation. [82] [83]

ABNORMALITIES OF BONE TURNOVER

Chronic kidney disease is associated with a variety of bone disorders and disorders of calcium and phosphorus metabolism. The major disorders of bone are associated with high parathyroid hormone levels.

Vascular and Soft Tissue Calcification

In CKD extra skeletal calcification is highly prevalent. Vascular calcification prevalence in dialysis patients ranges from 50-90% in more than 20 studies that have addressed these using different modalities and is even present in children on dialysis therapy. Vascular calcification appears to start early in CKD and 50% of patients initiated on hemodialysis (HD) therapy already have evidence of coronary artery calcification (CAC). Age and dialysis vintage are consistently associated with coronary artery calcification. There are two types of vascular calcification: Intimal calcification - calcific plaques or circumferentially calcified atherosclerosis. Medial calcification is nonocclusive and leads to vascular stiffening; it can cause local ischemia and also affect the capacity of the vasculature to dampen increases in arterial pressure with each ventricular systole, leading to left ventricular hypertrophy.

Pathogenesis of vascular calcification:

It features a phenotypic switch in which vascular smooth muscle cells (VSMCs) dedifferentiate to osteo/chondrocytic-like cells. The most important stimulus appears to be hyperphosphatemia, but others such as inflammation, cytokines, oxidative stress and advanced glycation end products also can enhance this transformation. Osteo/chondrocytic-like cells lay down collagen and noncollagenous proteins in the intima or media. Calcium and phosphorus are incorporated into matrix vesicles to initiate mineralization in the form of hydroxyapatite.

When the balance favours pro-mineralizing factors (e.g., elevations in calcium and phosphorus) over inhibitors of calcification (e.g., fetuin A, matrix GLA protein, osteopontin and pyrophosphate) calcification occurs. Patients with CKD who have low-turnover bone disease appear to have the greatest risk of vascular calcification. It is likely that adynamic bone is not able to take up high calcium loads and this excess calcium may become deposited in the vasculature.

MATERIALS & METHODS

4. MATERIALS AND METHODS

Source of Data

This study was duration based case control study, carried out for a period of six months on clinically confirmed cases of CKD visiting Nephrology OPD and in patients at Chennai Medical College Hospital & Research Centre, Irungalur. It also included 50 healthy controls visiting for regular health check-up. Both, males and females were included in the study.

Inclusion Criteria:

Patients in various stages of CKD (Age above 18years)

Exclusion Criteria:

Patients with autoimmune disorders,

Post parathyroidectomy status patients.

Collection of Sample:

After obtaining approval from ethical committee and a written informed consent from both cases and controls, samples were collected. The baseline data, clinical history of both cases and controls were taken. The clinical findings, investigation report were entered on a predesigned proforma.

Under strict aseptic precautions, 5ml of venous blood was collected after 8-10 hours of overnight fasting, from both cases and controls.

Blood samples were collected in vaccutainer and kept at room temperature for 30-40 minutes for clotting and then centrifuged at 3000 revolutions per minute for 10 minutes. Serum samples were separated and collected in an Eppendorf tube and following investigations were carried out.

1) Intact PTH	-	Electrochemiluminescence immunoassay (ECLIA)
2) Serum calcium	-	Photometric test using arsenazo III
3) Phosphorus	-	Photometric UV test with endpoint determination
4) Blood urea	-	GLDH method : Fully Enzymatic method
5) Serum creatinine	-	Modified Jaffe's Method
6) Alkaline Phosphatase	-	Kinetic photometric test

ESTIMATION OF INTACT PTH

Method

Electrochemiluminescence immunoassay (ECLIA) method

Principle:

Sandwich principle. Total duration of assay: 18 minutes.

▪ 1st incubation: 50 µL of sample, a biotinylated monoclonal PTH-specific antibody, and monoclonal PTH-specific antibody labeled with a ruthenium complex form a sandwich complex.

▪ 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Reagents - working solutions

The reagent rack pack is labeled as PTH.

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:

Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-PTH-Ab~biotin (gray cap), 1 bottle, 7 mL:

Biotinylated monoclonal anti-PTH antibody (mouse) 2.3 mg/L;

Phosphate buffer 100 mmol/L, pH 7.0; preservative.

R2 Anti-PTH-Ab~Ru(bpy) (black cap), 1 bottle, 7 mL:

Monoclonal anti-PTH antibody (mouse) labeled with ruthenium complex 2.0 mg/L; phosphate buffer 100 mmol/L, pH 7.0; preservative.

Reagent handling

The reagents in the kit have been assembled into a ready for use unit that cannot be separated. All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the Elecsys reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:

Unopened at 2-8 °C:	Up to the stated expiration date
After opening at 2-8 °C:	12 weeks
On the analyzers:	8 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes.

K2-EDTA and K3-EDTA plasma.

Because of the short half life of PTH, it is recommended that, when serum is needed, the blood should be centrifuged immediately.

Preference should be given to EDTA plasma, as it is stable longer than serum.

Criterion: Method comparison serum versus plasma, slope 0.9-1.1 + intercept within < ± 2 x analytical sensitivity (LDL) + coefficient of correlation > 0.95.

Serum: Stable for 8 hours at 15-25 °C, 2 days at 2-8 °C, 6 months at -20 °C.

Plasma: Stable for 2 days at 15-25 °C, 3 days at 2-8 °C, 6 months at -20 °C.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all

manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use samples and controls stabilized with azide.

Ensure the samples, calibrators and controls are at 20- 25 °C prior to measurement.

Due to possible evaporation effects, samples, calibrators and controls on the analyzers should be analyzed / measured within 2 hours.

Calibration

Traceability:

This method has been standardized against a commercial PTH test (RIA). The recovery of the NIBSC 95/646 (WHO) standard was assessed by testing dilutions in human serum covering the measuring range (40-4000 pg/mL) on 16 analyzers (**cobas e** 411 and **cobas e** 601 analyzers). The mean recovery was 100 % ± 4 %.

Every Elecsys reagent set has a barcoded label containing specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency:

Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer). Renewed calibration is recommended as follows:

- After 12 weeks when using the same reagent lot
- After 7 days (when using the same reagent kit on the analyzer)
- As required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Varia.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in pg/mL or pmol/L).

Conversion factors: pg/mL x 0.106 = pmol/L

pmol/L x 9.43 = pg/mL

Limitations - interference

The assay is unaffected by icterus (bilirubin < 1112 µmol/L or < 65 mg/dL), lipemia (Intralipid < 1500 mg/dL) and biotin (< 205 nmol/L or < 50 ng/mL).

The assay is affected by hemolysis ≥ 0.15 g/dL. Do not analyze samples that show visible signs of hemolysis.

Criterion: Recovery within ± 10 % of initial value.

Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

No interference was observed from rheumatoid factors up to a concentration of 1500 IU/mL.

There is no high-dose hook effect at PTH concentrations up to 17000 pg/mL (1802 pmol/L).

In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

1.20-5000 pg/mL or 0.127-530 pmol/L (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 1.20 pg/mL (< 0.127 pmol/L). Values above the measuring range are reported as > 5000 pg/mL (> 530 pmol/L).

Lower limits of measurement

Lower detection limit of the test

Lower detection limit: 1.20 pg/mL (0.127 pmol/L)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1 + 2 SD, repeatability study, n = 21).

Dilution

Not necessary due to the broad measuring range.

Expected values

15-65 pg/mL (1.6-6.9 pmol/L) [84] [85]

ESTIMATION OF CALCIUM

METHOD

Photometric test using arsenazo III

PRINCIPLE

Calcium with arsenazo III at neutral pH yields a blue colored complex, whose intensity is proportional to the calcium concentration. Interference by magnesium is eliminated by addition of 8-hydroxyquinoline-5-sulfonic acid.

REAGENTS

Components and concentrations

Reagent:

Phosphate buffer	pH 7.5	50 mmol/L
8-hydroxyquinoline-5-sulfonic acid		5 mmol/L
Arsenazo III		120 µmol/L
Standard		10 mg/dL (2.5 mmol/L)

STORAGE INSTRUCTIONS AND REAGENT STABILITY

The reagent is stable up to the end of the indicated month of expiry, if stored at 2-8˚C and contamination is avoided. Do not freeze the reagent.

The standard is stable up to the end of the indicated month of expiry, if stored at 2-25˚C.

Reagent preparation

The reagent and the standard are ready-to-use.

Materials needed but not provided

NaCl solution 9 g/L.

General laboratory equipment.

Specimen

Serum, heparin plasma or urine

Do not use EDTA plasma.

Stability [86]

in Serum/Plasma: 7 days at 20 - 25 °C

3 weeks at 4 - 8 °C

8 months at -20 °C

Add 10 mL of concentrated HCl to 24 h urine and heat the specimen to dissolve calcium oxalate.

Assay Procedure

Wavelength	650 nm, Hg 623 nm (630 – 670 nm)
Optical path	1 cm
Temperature	20 – 25 °C / 37 °C
Measurement	against reagent blank

	Blank	**Sample / Standard**
Sample / Standard	-	10 µl
Dist. Water	10 µL	-
Reagent	1000 µl	1000 µl
Mix, incubate for 5 minutes & read absorbance against reagent blank		

Calculation

With standard or calibrator

$$\text{Calcium [mg/dL]} = \frac{\text{A Sample}}{\text{A Std / Cal}} \times \text{Conc. Std / Cal [mg/dL]}$$

CALIBRATORS AND CONTROLS:

TruCal U

TruLab N

TruLab P

Performance Characteristics

Measuring range

The test has been developed to determine calcium concentrations within a measuring range from 0.04 – 20 mg/dL (0.01 – 5 mmol/L). When values exceed this range samples should be diluted 1+1 with NaCl solution (9 g/L) and the result multiplied by 2.

Specificity / Interferences

No interference was observed by ascorbic acid up to 30 mg/dL, bilirubin up to 40 mg/dL, hemoglobin up to 500 mg/dL, lipemia up to 2,000 mg/dL triglycerides and magnesium up to 15 mg/dL. Strontium salts in medicine may lead to strongly increased calcium values.

Sensitivity / Limit of Detection

The lower limit of detection is 0.04 mg/dL (0.01 mmol/L).

Reference Range

Serum/Plasma [87] : 8.6 – 10.3 mg/dL (2.15 – 2.57 mmol/L)

ESTIMATION OF PHOSPHORUS

METHOD

Photometric UV test with endpoint determination

PRINCIPLE

Ammonium molybdate + Sulphuric acid + Phosphate

→ Inorg. Phosphorus molybdate complex

Maximum complex absorption is at 340 nm.

REAGENTS

Components and concentrations

R1:	Glycine/sulphuric acid buffer	50 mmol/L
R2:	Glycine buffer	50 mmol/L
	Ammonium molybdate	1.75 mmol/L
Standard (Phosphorus):		5 mg/dL (1.61 mmol/L)

REAGENT STORAGE AND STABILITY

The reagents and standard are stable up to the end of the indicated month of expiry, if stored at 2-8˚C and contamination is avoided. Do not freeze the reagents.

Reagent preparation

The reagent and the standard are ready-to-use.

Materials needed but not supplied with reagent kit

NaCl solution 9 g/L.

General laboratory equipment.

Specimen

Serum, heparin plasma or urine

Stability in serum/plasma: 1 day at 20 – 25 °C

4 days at 4 – 8 °C

1 year at -20 °C

Assay Procedure

Wavelength	340 nm, Hg 334 nm, Hg 365 nm
	660 nm bichromatic
Optical path	1 cm
Temperature	20 - 25 °C/37 °C
Measurement	Against reagent blank

SUBSTARTE START PROCEDURE:

	Blank	**Sample / Standard**
Sample / Standard	-	10 µL
Dist. Water	10 µL	-
Reagent 1	800 µL	800 µL
Mix, incubate 5 minutes., read absorbance A1, then add:		
Reagent 2	200µL	200µL
Mix, read absorbance A2 within 5 - 60 minutes. Calculate the absorbance difference: ΔA = [(A2 – A1) Sample / Standard]		

SAMPLE START PROCEDURE:

	Blank	**Sample / Standard**
Sample / Standard	-	10 µl
Dist. Water	10 µL	-
Monoreagent	1000 µl	1000 µl
Mix, incubate 5 minutes., read absorbance against reagent blank within 60 minutes. Calculate the absorbance difference: ΔA = A Sample / Standard		

Calculation

With standard or calibrator

$$\text{Phosphorus [mg/dL]} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Std / Cal}} \times \text{Conc. Std / Cal [mg/dL]}$$

CALIBRATORS AND CONTROLS:

TruCal U

TruLab N

TruLab P

TruLab Urine Level 1

TruLab Urine Level 2

Performance Characteristics

Measuring range

The test has been developed to determine phosphorus concentrations within a measuring range from 0.2– 30 mg/dL (0.065 – 9.69 mmol/L). When values exceed this range samples should be diluted 1 + 10 with NaCl solution (9 g/L) and the result multiplied by 11.

Specificity/Interferences

No interference was observed by ascorbic acid up to 30 mg/dL, bilirubin up to 60 mg/dL, hemoglobin up to 1000 mg/dL and lipemia up to 2000 mg/dL triglycerides. Please be aware that ditaurobilirubin interferes from the concentration 3 mg/dL on, when phosphate is measured on systems which are unable to handle a second wavelength. For further information on interfering substances refer to Young DS [5].

Sensitivity/Limit of Detection

The lower limit of detection is 0.2 mg/dL (0.065 mmol/L).

Reference Range

Serum/Plasma [88]: Adults 2.6 - 4.5 mg/dL

ESTIMATION OF BLOOD UREA

METHOD

Glutamate Dehydrogenase (GLDH) method : Fully Enzymatic method

PRINCIPLE

$$\text{Urea} + 2\ H_2O \xrightarrow{\text{----Urease---}} 2\ NH_4^+ + Co_3^{2-}$$

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{----GLDH---}} \text{L-glutamate} + H_2O + NAD^+$$

CONTENTS

ENZ	8 x 40 ml Enzymes	
	Tris buffer (pH 7.8)	125 mmol/L
	ADP	0.88 mmol/L
	Urease	≥ 20 kU/L
	GLDH	≥ 0.3 kU/L
	Sodium Azide	0.095 %
SUB	8 x 10 ml Substrate	
	2-Oxoglutarate	25 mmol/L
	NADH	1.25 mmol/L
	Sodium Azide	0.095 %
STD	1 x 3 ml Standard	
	Urea	80 mg/dL (13.3 mmol/L)
	Sodium Azide	0.095 %

REAGENT PREPARATION

The reagents are ready for use and can directly be applied on automated analyzers (Reagent start procedure).

For sample start procedure **working reagent** is prepared by mixing 4 parts of ENZ with 1 part of SUB; e.g. 40 ml ENZ + 10 ml SUB

REAGENT STORAGE AND STABILITY

The individual reagents are stable, even after opening, up to the end of the indicated month of expiry, if stored at 2-8˚C and contamination is avoided.

The working reagent is stable for 5 days at 15-25˚C and for 4 weeks at 2-8˚C.

SPECIMEN

Serum, plasma (except ammonium heparinate plasma)

Stability [89]

In serum/plasma:	7 days at 20 – 25 °C
	1 year at -20 °C

Assay

Wavelength	340 nm, Hg 334 nm, 365nm
Optical path	1 cm
Temperature	25° C, 30° C or 37° C
Measurement	Against reagent blank

PROCEDURE:

Reagent Start Procedure

	Reagent Blank (RB)	**Sample / Standard**
Sample / Standard	-	10 µL
Enzyme	1000 µL	1000 µL
Mix, incubate for approximately for 1 minute.		
Substrate	250µL	250µL
Mix, read absorbance of Sample / Standard after 30 seconds (A_1) and read after exactly 1 minute (A_2). Calculate the absorbance difference: $\Delta A_{Sample / Standard} = (A_2-A_1) - \Delta A_{RB}$.		

SAMPLE START PROCEDURE:

	Reagent blank (RB)	**Sample / Standard**
Sample / Standard	-	10 µl
Working reagent	1000 µl	1000 µl
Mix, read absorbance of Sample / Standard after 30 seconds (A_1), and read again after exactly 1 minute (A_2). Calculate the absorbance difference: $\Delta A_{Sample / Standard} = (A_2-A_1) - \Delta A_{RB}$.		

CALCULATION:

Serum, Plasma

$$C = 80.0 \times \Delta A_{Sample} / \Delta A_{Standard} \text{ [mg/dL]}$$

or

$$C = 13.3 \times \Delta A_{Sample} / \Delta A_{Standard} \text{ [mmol/L]}$$

LINEARITY:

The test is linear up to 300 mg/dL or 50 mmol/L.

REFERENCE VALUES:

Serum [90]: 10 - 50 mg / dL or 1.7 - 8.3 mmol / L

QUALITY CONTROL

All control sera with urea values determined by this method can be employed.

ESTIMATION OF CREATININE

METHOD

Kinetic test without deproteinization according to Jaffe method

Principle

Creatinine forms a colored orange-red complex in an alkaline picrate solution. The difference in absorbance at fixed times during conversion is proportional to the concentration of creatinine in the sample.

Creatinine + Picric acid → Creatinine picrate complex

REAGENTS

Components and concentrations

R1:	Sodium hydroxide	0.2 mol/L
R2:	Picric acid	20 mmol/L
Standard:		2 mg/dL (177 µmol/L)

REAGENT STORAGE AND STABILITY

The reagents and standard are stable up to the end of the indicated month of expiry, if stored at 2-25 ˚ C and contamination is avoided. Do not freeze the reagents and protect from direct light.

Reagent preparation

The standard is ready-to-use.

Substrate start

The reagents are ready-to-use.

Sample start

Mix 4 parts of R1 + 1 part of R2;

Stability: 5 hours at 15 - 25˚C

Materials needed but not supplied with reagent kit

NaCl solution 9 g/L.

General laboratory equipment.

Specimen

Serum, heparin plasma, urine

Stability

In serum / plasma: 7 days at 4-25°C

at least 3 months at -20°C

Assay Procedure

Wavelength Hg 492 nm, (490 - 510 nm)

Optical path 1 cm

Temperature 20 - 25 °C / 37 °C

Measurement Against reagent blank

PROCEDURE:

SUBSTARTE START PROCEDURE:

	Blank	**Sample / Standard**
Sample / Standard	-	50 µL
Dist. Water	50 µL	-
Reagent 1	1000 µL	1000 µL
Mix, incubate 0-5 minute., then add:		
Reagent 2	250µL	250µL
Mix, read absorbance after 60 seconds (A_1) and read after further 2 minutes (A_2). Calculate the absorbance difference: ΔA = [(A2 – A1) sample or standard] - [(A2 – A1) blank]		

SAMPLE START PROCEDURE:

	Blank	**Sample / Standard**
Sample / Standard	-	50 µl
Dist. Water	50 µL	-
Monoreagent	1000 µl	1000 µl
Mix, read absorbance after 60 seconds (A_1) and read after further 2 minutes (A_2). Calculate the absorbance difference: ΔA = [(A2 – A1) sample or standard] - [(A2 – A1) blank]		

Calculation

With standard or calibrator

Serum/Plasma

Creatinine [mg / dL] = $\Delta A_{Sample} / \Delta A_{Standard / Cal}$ x Conc. Std /Cal [mg / dL]

CALIBRATORS AND CONTROLS:

TruCal U

TruLab N

TruLab P

TruLab Urine Level 1

TruLab Urine Level 2

Performance Characteristics

Measuring range

The test has been developed to determine creatinine concentrations within a measuring range from 0.2 – 15 mg/dL (18 – 1330 µmol/L). When values exceed this range samples should be diluted 1 + 1 with NaCl solution (9 g/L) and the result multiplied by 2.

Sensitivity/Limit of Detection

The lower limit of detection is 0.2 mg/dL.

Specificity/Interferences

No interference was observed by ascorbic acid up to 30 mg/dL, hemoglobin up to 500 mg/dL and lipemia up to 2,000 mg/dL triglycerides. Bilirubin interferes starting with a bilirubin concentration of 4 mg/dL.

Reference Range [91]

Men:	0.7 – 1.2 mg/dL
Women:	0.5 – 0.9 mg/dL

ESTIMATION OF ALKALINE PHOSPHATASE

METHOD

Kinetic photometric test

PRINCIPLE

p-Nitrophenylphosphate + H2O ----AP→ Phosphate + p-Nitrophenol

REAGENTS

Components and concentrations

R1:	2-Amino-2-methyl-1-propanol	pH 10.4	1.1mol/L
	Magnesium acetate		2 mmol/L
	Zinc sulphate		0.5 mmol/L
	HEDTA		2.5 mmol/L
R2:	p-Nitrophenylphosphate		80 mmol/L

REAGENT STORAGE AND STABILITY

The reagents and standard are stable up to the end of the indicated month of expiry, if stored at 2-8˚C and contamination is avoided. Do not freeze the reagents and reagent 2 must be protected from light.

Materials needed but not supplied with reagent kit

NaCl solution 9 g/L.

General laboratory equipment.

Reagent preparation

Substrate start

The reagents are ready-to-use.

Sample start

Mix 4 parts of R1 + 1 part of R2;

Stability: 5 days at 15 - 25˚C; 4 weeks at 2 - 8˚C;

The monoreagent must be protected from light.

Specimen

Serum or heparin plasma. Do not use hemolytic samples.

Stability [92]: 7 days at 4 – 8 °C

2 months at - 20 °C

Assay Procedure

Wavelength	Hg 405 nm, (400 – 420 nm)
Optical path	1 cm
Temperature	37 °C
Measurement	Against reagent blank

SUBSTARTE START PROCEDURE:

	Blank	**Sample or Calibrator**
Sample or Calibrator	-	20 µL
Dist. Water	20 µL	-
Reagent 1	1000 µL	1000 µL
Mix, incubate for 1 minute., then add:		
Reagent 2	250µL	250µL
Mix, read absorbance after 1 minute and read again after 1, 2 & 3 minutes.		

SAMPLE START PROCEDURE:

	Blank	**Sample or Calibrator**
Sample or Calibrator	-	20 µl
Dist. Water	20 µL	-
Monoreagent	1000 µl	1000 µl
Mix, read absorbance after 1 minute and read again after 1, 2 & 3 minutes.		

Calculation

With factor

From absorbance readings calculate ΔA/min and multiply by the corresponding factor from table below:

ΔA/min x factor = AP activity [U/L]

Substrate start	405 nm	3433
Sample start	405 nm	2757

With calibrator

$$AP\ [U/L] = \frac{\Delta A / min\ Sample}{\Delta A / min\ Calibrator} \times Conc.\ Calibrator\ [U/L]$$

Calculation factor

ALP [U/L] x 0.0167 = ALP [µkat/L]

CALIBRATORS AND CONTROLS:

TruCal U

TruLab N

TruLab P

Performance characteristics

Measuring range

On automated systems the test is suitable for the determination of AP activities up to 1400 U/L. In case of a manual procedure, the test is suitable for AP activities which correspond to a maximum of ΔA/min of 0.25. If such values are exceeded the samples should be diluted 1 + 9 with NaCl solution (9 g/L) and results multiplied by 10.

Specificity/Interferences [93]

No interference was observed by ascorbic acid up to 30 mg/dL, conjugated bilirubin up to 60 mg/dL, unconjugated bilirubin to 25 mg/dL, hemoglobin up to 100 mg/dL and lipemia up to 2000 mg/dL triglycerides.

Sensitivity/Limit of Detection

The lower limit of detection is 2 U/L.

Reference Range [94]

Men	:	40 – 130 [U/L]
Women	:	35 – 105 [U/L]

RESULTS

5. RESULTS

Statistical Methods:

Descriptive and inferential statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean±SD (Min-Max) and results on categorical measurements are presented in Number (%). Significance is assessed at 5 % level of significance. The following assumptions on data are made; Assumptions: 1. Dependent variables should be normally distributed, 2. Samples drawn from the population should be random, and 3. Cases of the samples should be independent.

Analysis of variance (ANOVA) has been used to find the significance of study parameters between three or more groups of patients, Student test (two tailed, independent) has been used to find the significance of study parameters on continuous scale between two groups Inter group analysis) on metric parameters. Leven1s test for homogeneity of variance has been performed to assess the homogeneity of variance. Chi square/Fisher Exact test has been used to find the significance of study parameters on categorical scale between two or more groups. [95] [96] [97] [98]

Significant figures

** Strongly significant (P value: $P \leq 0.01$)

* Moderately significant (P value: $0.01 < P \leq 0.05$)

+ Suggestive significance (P value: $0.05 < P < 0.10$)

Statistical analysis: Data were analysed using SPSS 20.0.; Microsoft word and Excel have been used to generate graphs, tables**.**

In our study of role of intact parathyroid hormone and other biochemical parameters in early diagnosis of mineral disturbances in patients with Chronic Kidney Disease, the following results were obtained for 50 cases and 50 controls. Comparison of biochemical parameters in two groups studied was as follows;

The table below summarizes the findings, which are elaborated later on.

Biochemical parameters	Cases	Controls	P value
PTHpg/ml	136.80±92.70	52.47±16.34	<0.001**
Urea mg/dl	76.60±69.77	23.54±7.46	<0.001**
Creatinine mg/dl	4.11±4.25	0.56±0.10	<0.001**
Calcium mg/dl	8.35±1.07	8.98±0.76	0.001**
Phosphorus mg/dl	4.40±1.70	3.47±0.62	0.001**
Alkaline Phophatase IU/L	90.92±46.37	82.91±21.78	0.285

Table 1: Comparison of bio chemical parameters in cases and controls

Age in years	Cases		Controls	
	No	%	No	%
20-30	4	8	6	12
31-40	12	24	14	28
41-50	10	20	13	26
51-60	16	32	11	22
61-70	8	16	6	12
Total	50	100.0	50	100.0
Mean ± SD	47.26±12.73		43.83±15.12	

Table 2: Age distribution in cases and controls

Samples were age matched.

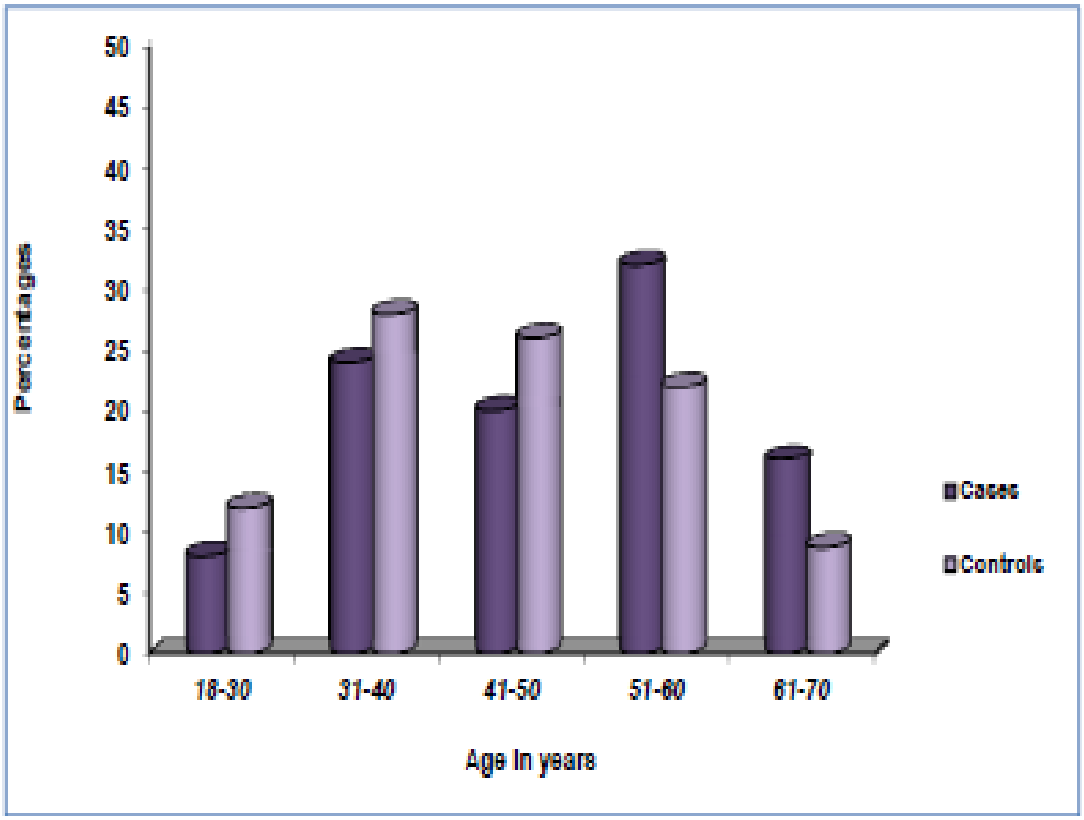

Fig 6: Age distribution in cases and controls

Samples were matched according to their age. Maximum number of patients, 32% were in the age group of 51-60 years, followed by 24% patients in 31-40 years. The mean age in cases is 47.26 ±12.73 years, whereas in controls mean age is 43.83 ±15.12 years.

Gender	Cases		Controls	
	No	%	No	%
Male	35	70	31	62
Female	15	30	19	38
Total	50	100.0	50	100.0

Table 3: Gender distribution in cases and controls studied

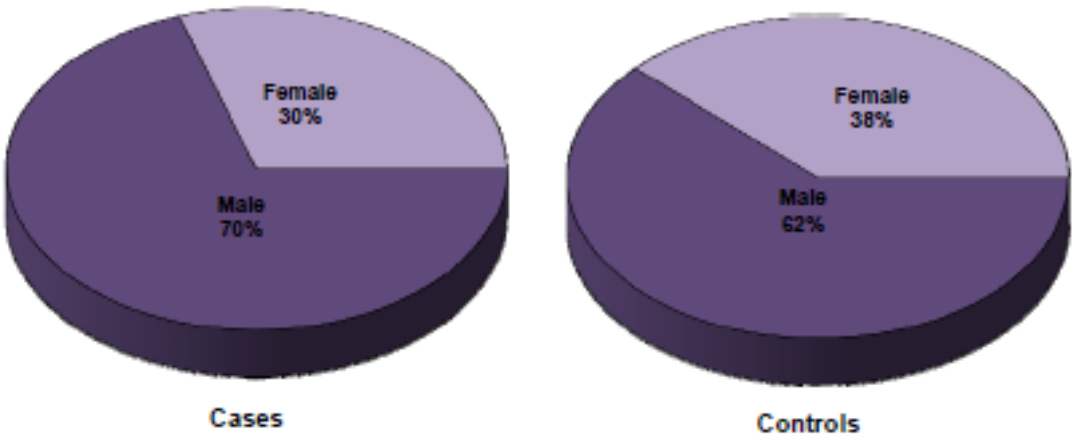

Fig 7: Gender distribution in cases and controls studied

Samples are gender matched. 70% of cases are males and 30% are females. In controls 62% are males and 38% are females.

Distribution of PTH in two groups studied

Parathyroid hormone levels were compared in both cases and controls. The mean level of PTH in cases is 136.80 ±92.70 where as in controls mean PTH level is 52.47 ±16.34 pg/mL. Statistically significant increase in levels of PTH were seen in patients than controls (P<0.001).

PTH pg/ml	Cases		Controls	
	No	%	No	%
<67	15	30	46	92
67-90	13	26	3	6
>90	22	44	1	2
Total	50	100.0	50	100.0
Mean ± SD	136.80±92.70		52.47±16.34	
Inference	PTH values is significantly higher in cases compared to controls with p<0.001**			

Table 4: Distribution of PTH in two groups studied

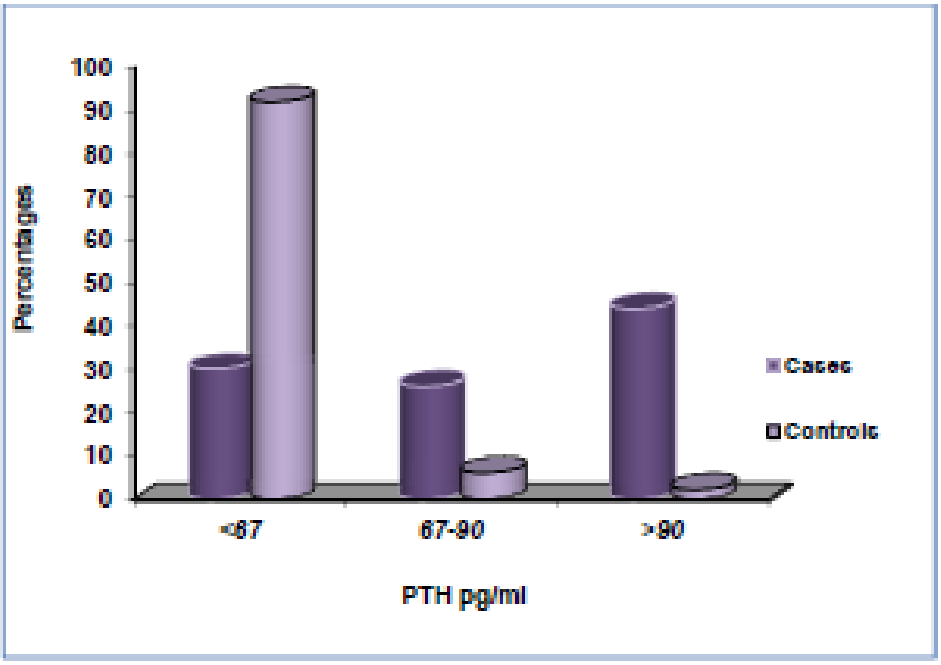

Fig 8: Distribution of PTH in two groups studied

Distribution of Urea in two groups studied

Urea levels were measured in cases and controls. The normal reference range of urea is 18-46 mg/dL. 52% of patients in CKD had urea levels above 46 mg/dL, none of the controls had urea levels above 46 mg/dL.

36% of patients and 74% of controls had urea levels between 18-46 mg/dL. 26% of controls and 12% of cases had urea levels <18 mg/dL.

Bio chemical parameter	Cases (n=50)		Controls (n=50)	
	No	%	No	%
Urea mg/dl				
• <18	6	12	13	26
• 18-46	18	36	37	74
• >46	26	52	0	0.0

Table 5: Distribution of Urea in two groups studied

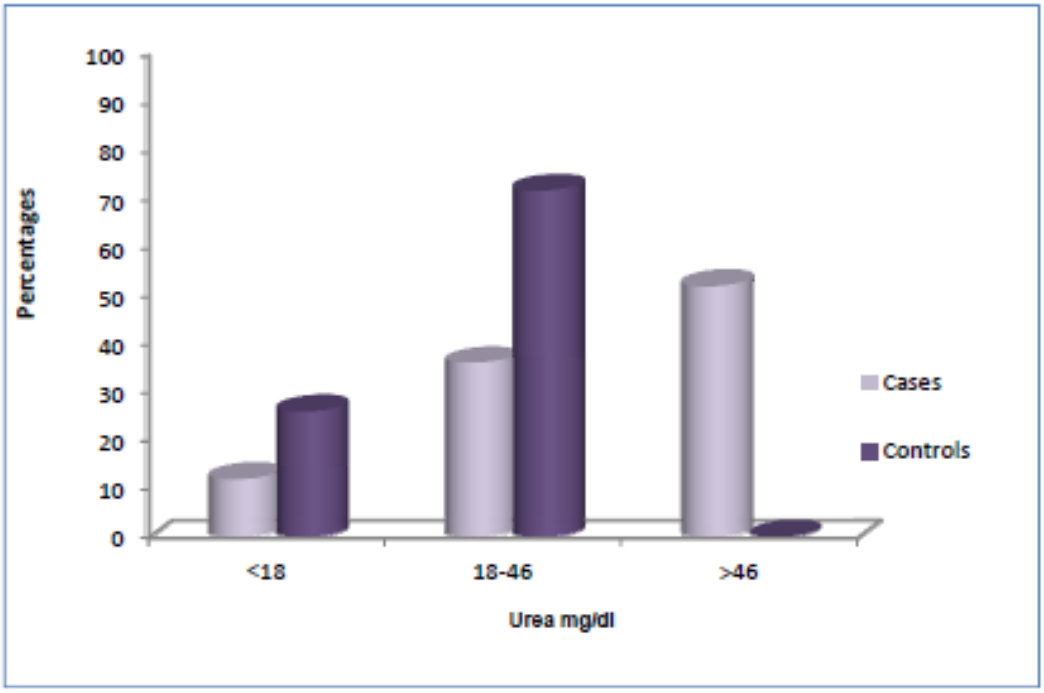

Fig 9: Distribution of Urea in two groups studied

Distribution of Creatinine in two groups studied

Creatinine levels were measured in cases and controls. The normal reference range for creatinine is between 0.6-1.2 mg/dL. 54% of cases had Creatinine levels >1.2mg/dL and none of the controls were in this range, 46% of case had Creatinine levels between 0.6-1.2 mg/dL and 48% of controls had the values in same range. 52% of controls had levels less <0.6 mg/dL and none of cases had within this range.

Bio chemical parameter	Cases (n=50)		Controls (n=50)	
	No	%	No	%
Creatinine mg/dl				
• <0.6	0	0.0	26	52
• 0.6-1.2	23	46	24	48
• >1.2	27	54	0	0.0

Table 6: Distribution of Creatinine in two groups studied

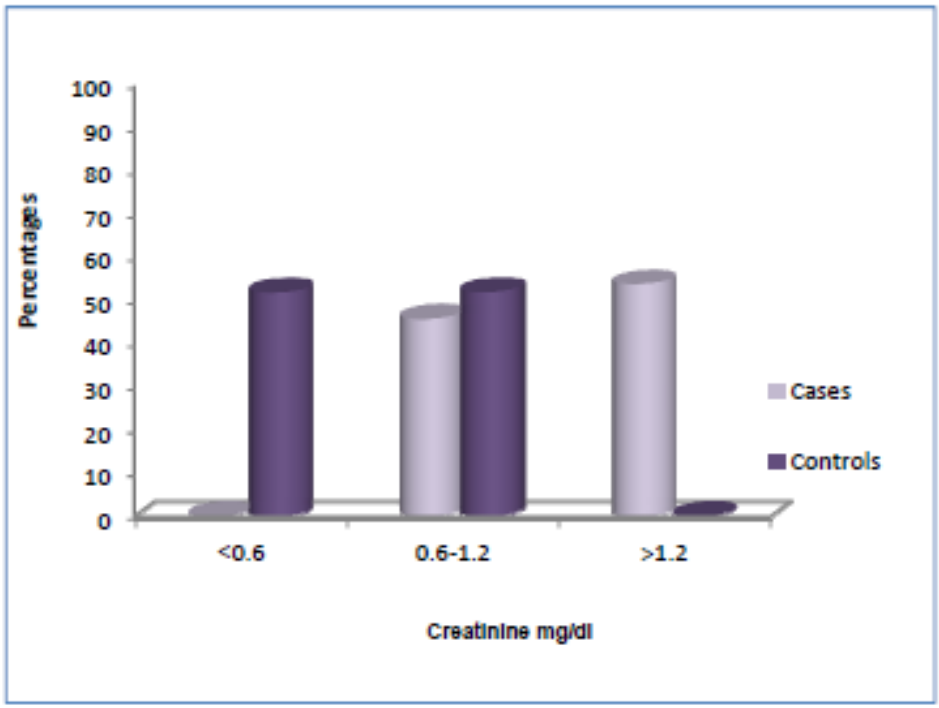

Fig 10: Distribution of Creatinine in two groups studied

Distribution of Calcium in two groups studied

Calcium levels were compared in cases and controls. The normal reference range of calcium is 8.5-10.2 mg/dL. 51.9% of cases had calcium levels between 8.5-10.2 mg/dL and 76% of controls had within the same range where as 48.1% of cases and only 24% of controls had levels <8.5 mg/dL.

Bio chemical parameter	*Cases (n=50)*		*Controls (n=50)*	
	No	%	No	%
Calcium mg/dl				
• <8.5	24	48.1	12	24.0
• 8.5-10.2	26	51.9	38	76.0
• >10.2	-	-	-	-

Table 7: Distribution of Calcium in two groups studied

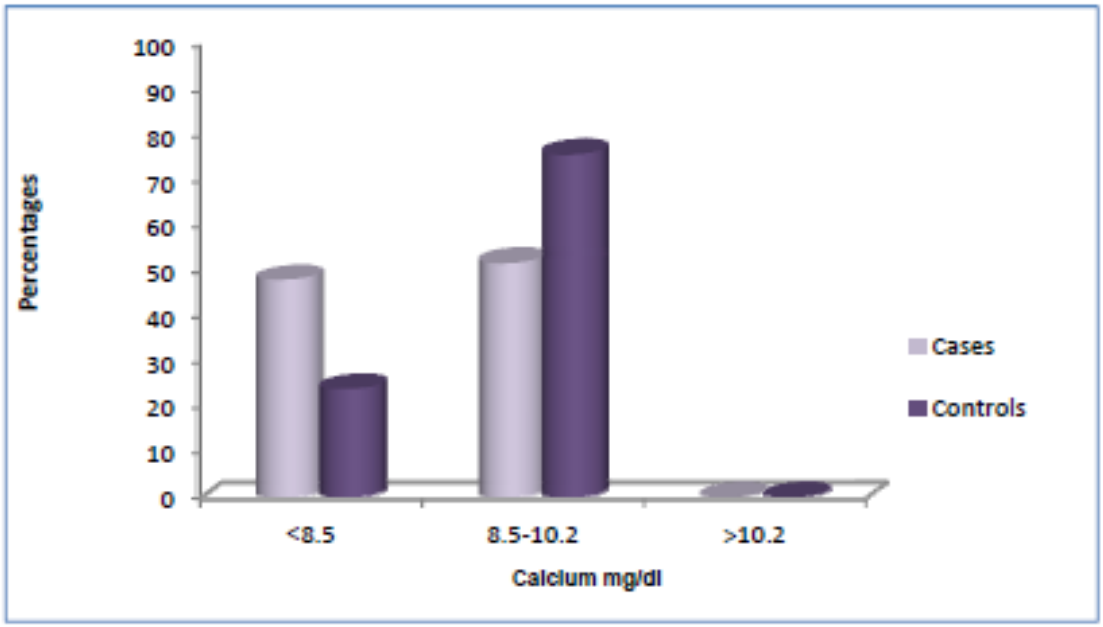

Fig 11: Distribution of Calcium in two groups studied

Distribution of Phosphorus in two groups studied

Phosphorus levels were measured in cases and controls. The normal reference range of Phosphorus is 2.5-4.5 mg/dL. 62% of cases had Phosphorus levels between 2.5-4.5mg/dL and 98% of controls.20% of cases had levels between 4.6-5.5 mg/dL only 2% of controls had in same range. 18% of patients had levels >5.5 mg/dL and none of controls had in this range.

Bio chemical parameters	Cases (n=50)		Controls (n=50)	
	No	%	No	%
Phosphorus mg/dl				
• <2.5	4	8	5	10
• 2.6-3.5	14	28	21	42
• 3.6-4.5	13	26	23	46
• 4.6-5.55	10	20	1	2
• >5.5	9	18	0	0.0

Table 8: Distribution of Phosphorus in two groups studied

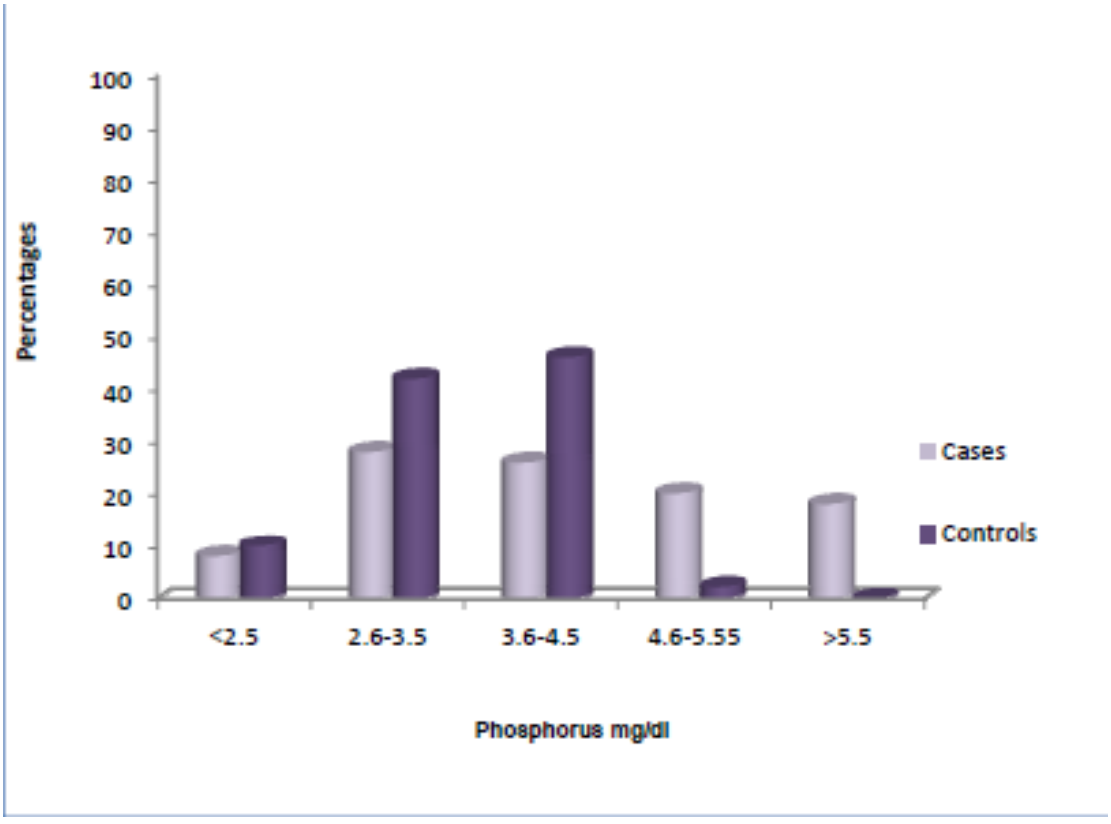

Fig 12: Distribution of Phosphorus in two groups studied

Distribution of Alkaline Phosphatase in two groups studied

Alkaline Phosphatase levels were measured in cases and controls. The normal range is 56-153 IU/L. 94% of patients had alkaline phosphatase levels between 56-153 IU/L where as 98% of controls had in the same range. 6% of cases had levels >153 IU/L and only 2% of controls had in the same range.

Bio chemical parameters	Cases (n=50)		Controls (n=50)	
	No	%	No	%
Alkaline Phosphatase IU/L				
• <56	6	12	5	10
• 56-153	41	82	44	88
• >153	3	6	1	2

Table 9: Distribution of Alkaline Phosphatase in two groups studied

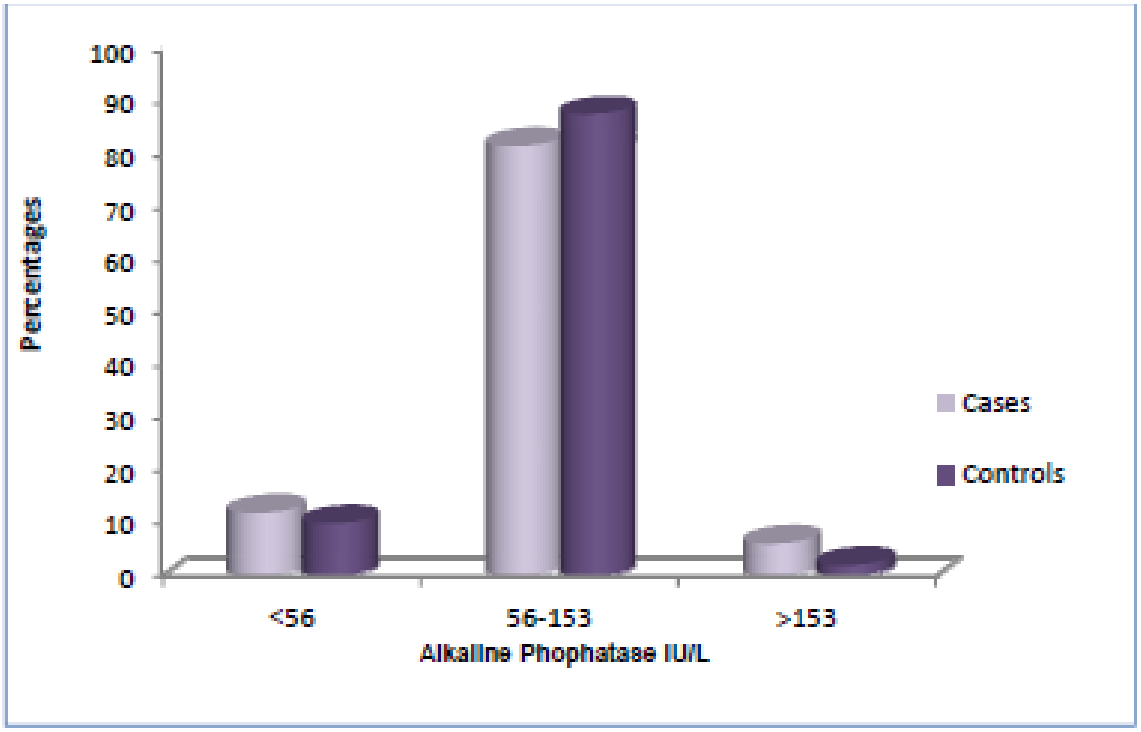

Fig 13: Distribution of Alkaline Phosphatase in two groups studied

Graphical representation of the findings

This is a table showing the mean levels of PTH, Urea, Creatinine, Calcium, Phosphorus and Alkaline phosphatase levels in cases and controls.

Biochemical parameters	Cases	Controls	P value
PTH pg/ml	136.80±92.70	52.47±16.34	<0.001**
Urea mg/dl	76.60±69.77	23.54±7.46	<0.001**
Creatinine mg/dl	4.11±4.25	0.56±0.10	<0.001**
Calcium mg/dl	8.35±1.07	8.98±0.76	0.001**
Phosphorus mg/dl	4.40±1.70	3.47±0.62	0.001**
Alkaline Phosphatase IU/L	90.92±46.37	82.91±21.78	0.285

Table 10: Comparison of mean levels of biochemical parameters in cases and controls

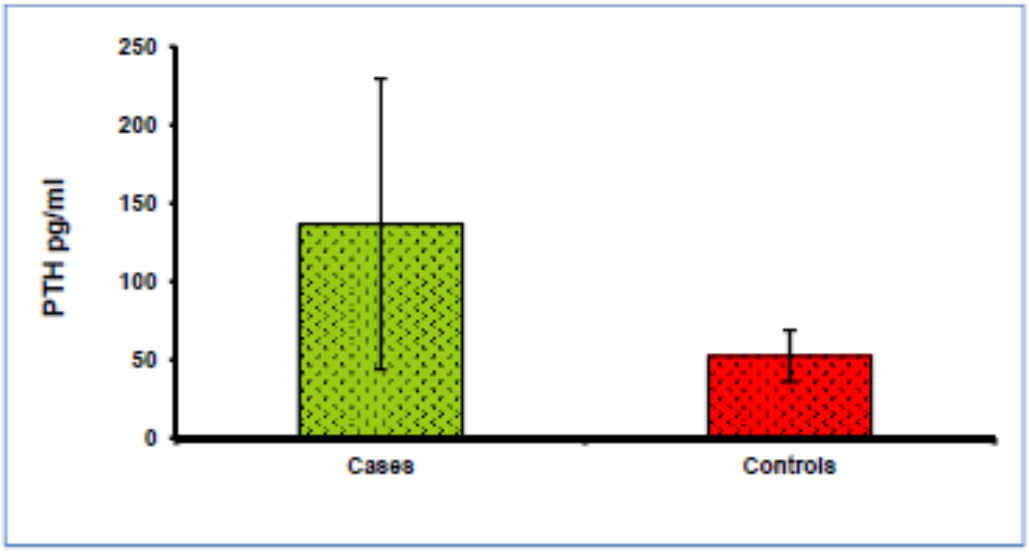

Fig 14: Mean PTH levels in cases and controls

The mean levels of **PTH** in cases are 136.80±92.70 and controls are 52.47±16.34 pg/ml. There is a statistically significant increase in PTH levels in cases as compared to controls; p value is <0.001.

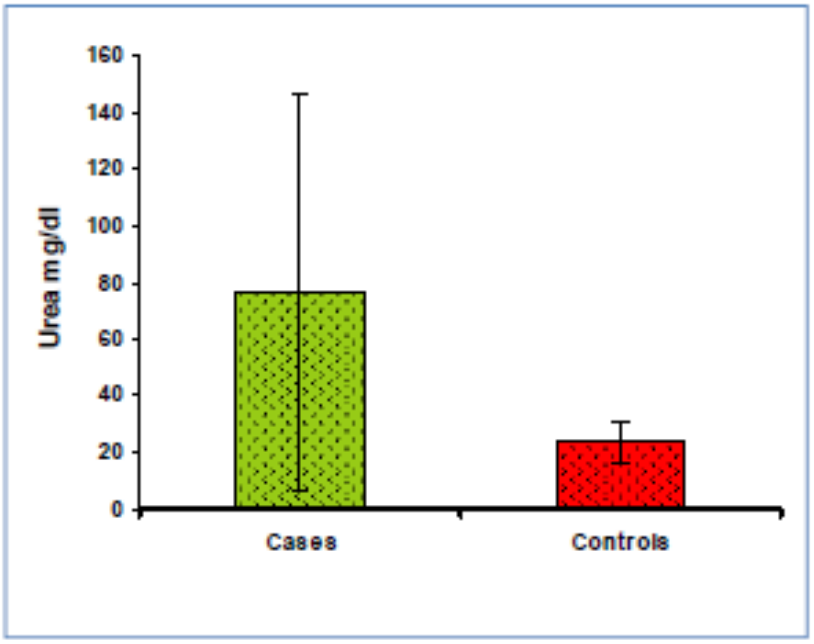

Fig 15: Mean Urea levels in cases and controls

Statistically significant increase in **urea** levels was seen in cases as compared to controls ($p<0.001$). The mean level in cases is 76.60±69.77 and control is 223.50± 7.46 ($p<0.001$).

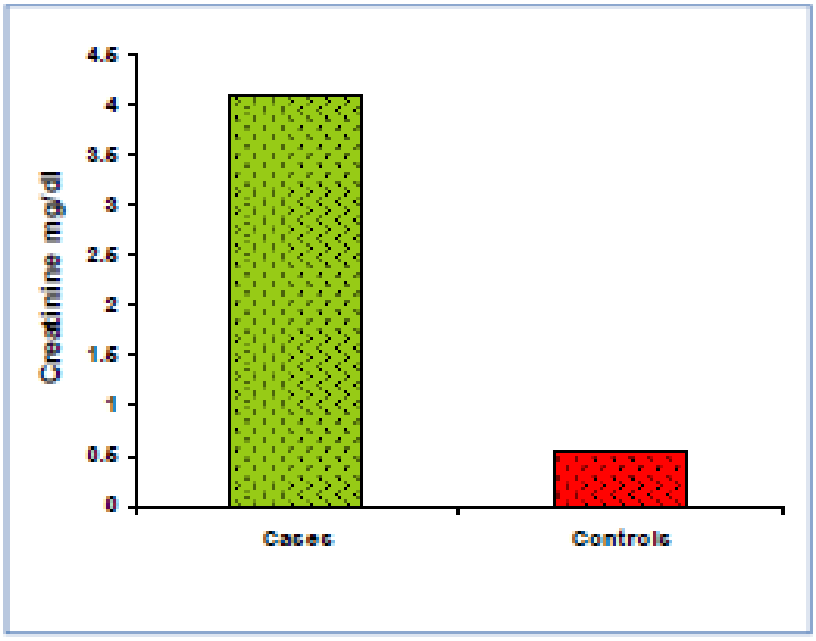

Fig 16: Mean Creatinine levels in cases and controls

Statistically significant increase in **creatinine** levels was seen in cases as compared to controls. The mean level in cases is 4.11±4.25 and control is 0.56±0.10 ($p<0.001$).

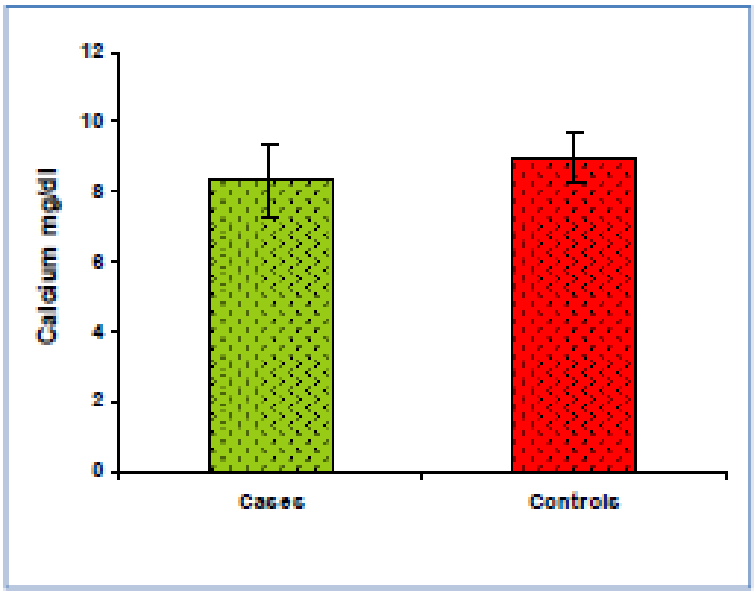

Fig 17: Mean Calcium levels in cases and controls.

Statistically significant increase in levels of **calcium** was seen in cases as compared to controls. The mean level of calcium in cases is 8.35±1.07 and control is 8.98±0.98 ($p<0.001$).

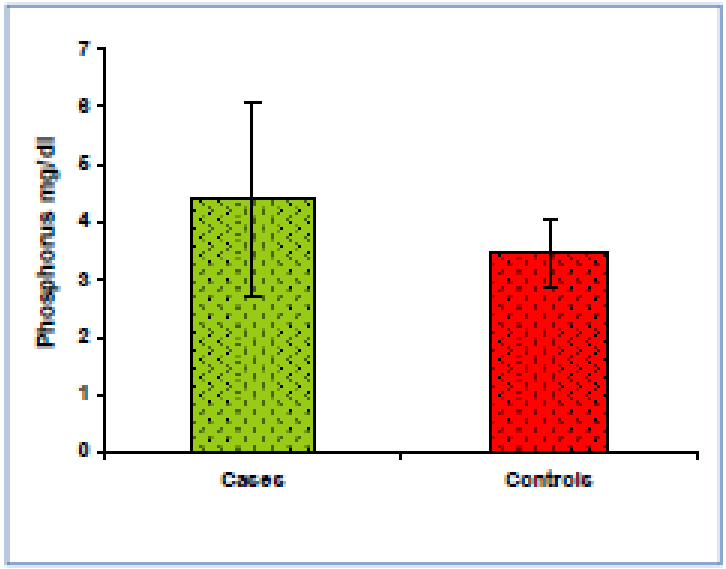

Fig 18: Mean Phosphorus levels in cases and controls

Increase in levels of **phosphorus** was observed in cases as compared to controls which was statistically significant. The mean level of phosphorus in cases is 4.40±1.70 and control is 3.47±0.62 ($p<0.001$).

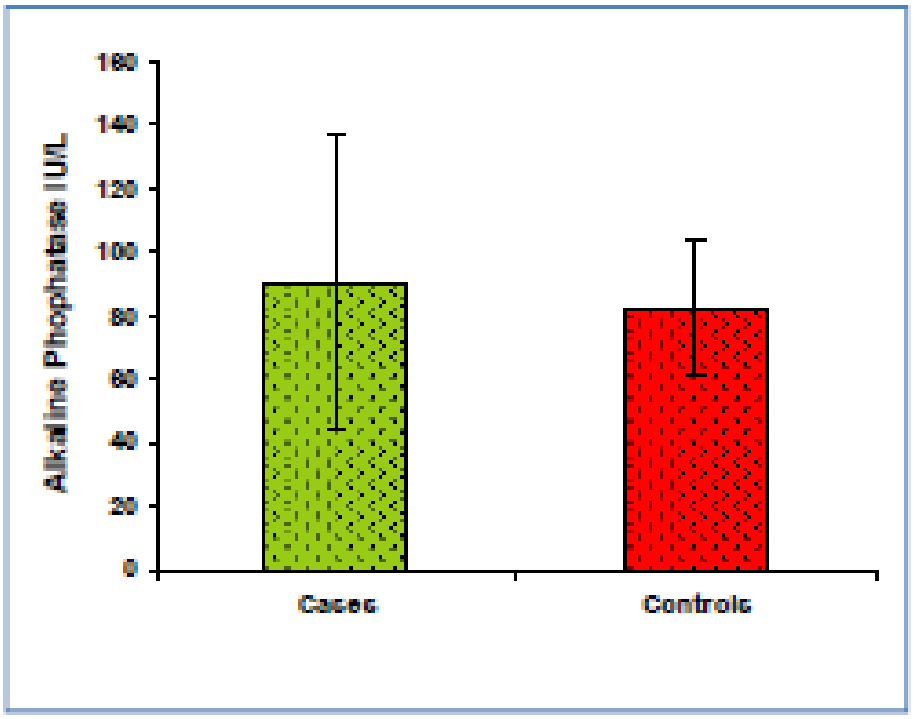

Fig 19: Mean Alkaline Phosphatase levels in cases and controls.

There is elevation in **Alkaline Phosphatase** levels in cases as compared to controls but the increase was not statistically significant. The mean level in cases is 90.92±46.37 and control is 82.91± 21.78 (p=0.285).

Distribution of cases in various stages of CKD were as follows

There were totally 50 cases; they were divided into various stages depending upon eGFR. 38% of patients were in CKD stage 5, followed by 36% in stage 2, 10% patients in stage 4 and 8% of patients were present in stage 1 and 3 respectively.

Stage of disease	Number of patients	%
Stage 1	4	8
Stage 2	18	36
Stage 3	4	8
Stage 4	5	10
Stage 5	19	38
Total	50	100.0

Table 11: Distribution of cases in various stages of CKD

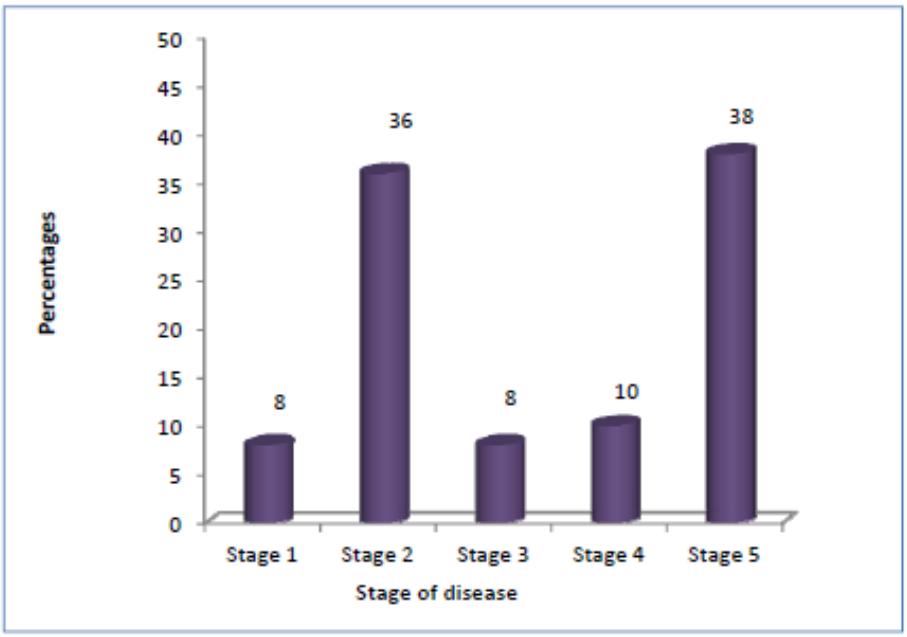

Fig 20: Distribution of cases in various stages of CKD

The levels of PTH, Calcium and phosphorus of patients in various stages of CKD are as follows:

In stage I CKD, we found that the levels of PTH, calcium and phosphorus were within normal range.

Test	Results
PTH pg/ml	67.72±29.92
Calcium mg/dl	8.3±1.01
Phosphorus mg/dl	3.25±0.62

In stage II CKD, the levels of PTH and phosphorus were slightly elevated; but within the reference range and calcium levels were also normal.

PTH pg/ml	75.09±33.38
Calcium mg/dl	8.48±1.12
Phosphorus mg/dl	3.99±0.76

In stage III, there is marginal elevation in PTH and phosphorus levels; and fall in calcium levels.

PTH pg/ml	96.47±33.88
Calcium mg/dl	7.43±1.46
Phosphorus mg/dl	5.55±1.72

In stage IV CKD, there is further elevation in PTH and phosphorus levels; and calcium levels come back to within range.

PTH pg/ml	158.98±115.1
Calcium mg/dl	8.50±0.91
Phosphorus mg/dl	5±2.8

In stage V CKD, there is significant increase in PTH levels, moderate increase in phosphorus levels and fall in calcium levels.

PTH pg/ml	211.13±88.0
Calcium mg/dl	8.38±1.01
Phosphorus mg/dl	4.66±2.07

The level of PTH, calcium and phosphorus are compared with various stages of CKD.

Variables	Stage of CKD				
	Stage I	Stage II	Stage III	Stage IV	Stage V
PTH pg/ml	67.72±29.92	75.09±33.38	96.47±33.88	158.98±115.1	211.13±88.0
Calcium mg/dl	8.3±1.01	8.48±1.12	7.43±1.46	8.50±0.91	8.38±1.01
Phosphorus mg/dl	3.25±0.62	3.99±0.76	5.55±1.72	5±2.8	4.66±2.07

Table 12: Values of PTH, Ca and P in different stages of CKD

DISCUSSION

6. DISCUSSION

Many studies and literature have shown that CKD is associated with alterations in calcium and phosphorus metabolism leading to increased mortality and morbidity. These alterations would cause changes in PTH levels in almost all stages of disease. We have used this in our study to find out the usefulness of the Elevated PTH levels as an early marker of derangements in bone and mineral metabolism associated with CKD. [99]

Nephrology guidelines also recommend targets and early treatment strategies to correct serum levels of phosphorus, calcium, and parathyroid hormone, because many data suggested there was a clear association between these potential risk biomarkers and vascular disease and death. [2] So numerous drugs including phosphorus binders, vitamin D and calcimimetic agents have been specifically developed and promoted to decrease these complications.

Pattern of Parathyroid hormone levels in CKD

Recent observational studies have shown that even a slight elevation in PTH levels has been associated with an increased cardiovascular risk. It is also found that monitoring PTH levels from the early stages of CKD can prevent complications due to mineral disturbances.

Elevated serum phosphorus has been associated with the progression of secondary hyperparathyroidism and deposition of calcium in soft tissues.

The long-term consequences associated with persistently elevated PTH levels in CKD include high-turnover bone disease, anemia, CVD, and mortality. [2] As a result, both NKF and Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend that PTH levels should be regularly monitored beginning in stage 3 CKD and that elevated levels should be treated with a combination of dietary phosphorus restriction and therapy with vitamin D and/or calcimimetic. [100]

In our study, we found a statistically significant increase in PTH level in cases as compared to controls ($p < 0.001$). The findings are similar to **Block et al** study, a significant increase in PTH levels was observed in CKD. They also identified high PTH levels as a significant correlate of all-cause mortality. They concluded that elevations in serum PTH might be associated with increased risk of death from cardiac causes. [79]

Amann K et al studies also implicated parathyroid hormone as a permissive factor that promotes cardiac fibroblast activation and intermyocardiocytic fibrosis. [101]

Wald et al, Tentori et al and **Kalantar-Zadeh et al** studies also observed a significant increase in PTH levels in CKD.

Phosphorus and Calcium in CKD:

Elevated serum phosphorus has been related to vascular & coronary artery calcification and resulting cardiovascular morbidity and mortality. Among mineral abnormalities; hyperphosphatemia is most prevalent among patients with ESRD.

Shanthi K et al studies have found that a significant increase in serum phosphorus levels in patients in CKD, [82] which is similar to that of our study. We observed a statistically significant increase in serum phosphorus levels in cases as compared to controls ($p < 0.001$).

Hyperphosphatemia and hypercalcemia have been shown to promote calcification of the vasculature, myocardium and cardiac valves. [74] Vascular calcification, manifested in reduced vessel wall elasticity, increased intima-media layer thickness and enhanced pulse-wave velocity, has been linked to LVH and occurs with increased severity in dialysis patients versus non-CKD patients. [102]

Craver et al studies observed a significant increase in phosphorus levels in patients who were in various stages of CKD ($p < 0.001$). He found that elevated PTH levels were associated with increased cardiovascular risk, loss of arterial elasticity and left ventricular hypertrophy. Relevant mechanism could be direct action on vascular and cardiac cells, which express PTH receptors.

J. Floege et al reported a significant increase in phosphorus levels and concluded that high level of phosphorus is a significant risk factor for mortality in CKD. [103]

Goodman et al in a resent observational study highlighted the increased prevalence and extent of coronary artery calcification in young dialysis patients compared with normal controls. [104]

Serum calcium levels are also involved in hyperparathyroidism progression but are more likely to play an important role in advanced stages, when they begin to decrease. **Schwartz et al** studies showed an association between higher levels of serum phosphorus and calcium-phosphorus product with an unfavourable renal outcome.[105] In their study higher serum phosphorus was associated with significantly higher risk for progression of CKD, even after adjustment for multiple potential confounders. The association of higher serum phosphorus with progressive CKD was more accentuated in patients with higher serum calcium. Thus supporting the hypothesis that tissue calcification may be the reason behind the complications. Tissue calcification is involved at the cellular and sub cellular levels, with hyperphosphatemia shown to be associated with increased expression of osteoblasts specific proteins in vascular smooth muscle cells (VSMCs). [106]

Reynolds et al showed that higher ambient serum calcium level led to more significant phosphorus-driven calcification of vascular smooth muscle in vitro. [107]

Rise in **urea** and **creatinine** levels are seen, which are used to support the diagnosis of CKD.

In our study, increase in alkaline phosphatase levels was observed in cases as compared to controls and the rise was of significant only in stage 5.

High serum **alkaline phosphatase** is associated with increased mortality. An analysis of the Dialysis Outcomes and Practice Patterns Study (DOPPS) database found that elevated serum alkaline phosphatase levels in hemodialysis patients were associated with higher risk of hospitalization and death.

The potential mechanisms for this observation remain unclear. A study by **Lee et al** concluded that, alkaline phosphatase can promote vascular calcification by hydrolyzing pyrophosphate in the arterial wall. [108]

Sigrist et al conducted a longitudinal study and found elevated levels of alkaline phosphatase in stage IV and V of CKD, they found that higher levels of serum alkaline phosphatase were associated with progressive arterial calcification. [109]

For early diagnosis, staging of CKD is required and was done by CKD-EPI equation and the levels of PTH, Calcium and Phosphorus of patients in various stages of CKD are as follows;

- ***In stage I*** *CKD, we found that the levels of PTH, calcium and phosphorus were within normal range.*
- ***In stage II*** *CKD, the levels of PTH and phosphorus were slightly elevated, but within the reference range; and calcium levels were also normal.*
- ***In stage III*** *CKD, there is marginal elevation in PTH and phosphorus levels; and fall in calcium levels.*
- ***In stage IV*** *CKD, there is further elevation in PTH and phosphorus levels; and calcium levels come back to within range.*
- ***In stage V*** *CKD, there is significant increase in PTH levels; moderate increase in phosphorus levels and fall in calcium levels.*

The findings are concordant with National Kidney Foundation's Kidney Early Evaluation Program (KEEP) study. [110] They observed a statistically significant increase in PTH, calcium & phosphorus levels in various stages of CKD. They found a significant decrease in levels of calcium from stage III to stage IV; whereas in stage V, it is again raised. Similar findings were observed in our study, where the levels of calcium is normal in stage I and II; the levels fall in stage III and come back to normal range in stage IV and V.

Kates DM et al, studies evaluated the relationships among serum phosphate, calcium, PTH and 1, 25-dihydroxyvitamin D in CKD patients who were in various stages of disease and demonstrated a similar finding. The study also suggested that phosphate may directly enhance PTH secretion in this setting. (111)

Levin et al performed a cross-sectional analysis and found that calcium and phosphorus values did not become abnormal and were relatively stable until stage IV CKD. (112) In our study we observed an increase in phosphorus levels from Stage III of CKD.

Until recently, it was thought that hyperphosphatemia was the earliest sign of SHPT and bone metabolism disorders. However, when patients reach Stage 3 CKD, it is highly probable that none of the routine biochemical parameters assessed will be abnormal. Infact, the PTH level is often increased before clinical hyperphosphatemia occurs.

Patel S et al, performed a cross-sectional study and observed that PTH levels increased with worsening of CKD. (113) A significant increase in PTH levels were also observed in our study which further increased with progression of CKD.

Levin A et al performed a cohort study in patients with stage 4–5 CKD and found that the levels of PTH and phosphorus were associated with an increased risk of death and the progression of renal failure, whereas vitamin D therapy was associated with better survival. (114)

Similar results indicating that secondary hyperparathyroidism is a risk factor associated with progression to dialysis or death have been obtained in cohort study conducted by **Schumock** CKD patients. (115)

CONCLUSION

7. CONCLUSION

The aim of our study was to correlate serum intact parathyroid hormone, urea, creatinine, calcium, phosphorus, alkaline phosphatase in patients with CKD and to compare it with controls and to know the role of parathyroid hormone in early diagnosis of mineral disturbances.

In our study, we found that statistically significant increase in the serum levels of intact parathyroid hormone, urea, creatinine, calcium and phosphorus, in patients with CKD as compared with controls. There was an increase in alkaline phosphatase level between cases and controls, but was not statistically significant. A statistically significant increase in PTH levels from stage III of CKD was also observed when calcium and phosphorus were still within normal range. Thus PTH levels can be used as a marker to identify the mineral disturbances in early stages of CKD.

Like in the standard guidelines which highlight the importance of measuring PTH early in the course of disease recommends an annual measurement of PTH once the diagnosis of CKD is made. If the PTH levels are measured and maintained within the target range, many complications can be prevented.

ANNEXURES

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PROFORMA

Name: Mr/Mrs. **Age:** Years; **Sex:** M / F ; **IP/OP No:**

Address: **Occupation:**

Ht: Cms; **Wt:** Kgs; **BMI:**

Complaints with H/O. Present illness:

H/O. Past illness:

Treatment History:

Duration of CKD/Stage:

Personal History: Smoking- ; Alcohol- ; Diet- Veg / Non Veg

Family History: (Diabetes / Hypertension / CAD / Thyroid disease)

O/E: Pulse- / min ; B.P- mmHg

S/E: CVS- ; RS- ; PA- ; CNS-

Investigations:

Urea (mg/dL)	
Creatinine (mg/dL)	
eGFR **(** mL/mt/1.73m^2**)**	
Calcium (mg/dL)	
Phosphorus (mg/dL)	
Alkaline Phosphatase (U/L)	
Intact PTH (pg/mL)	

CONSENT FORM - ENGLISH

Chennai Medical College Hospital & Research Centre
Irungalur, Trichy – 621 105.

Consent Form

You are requested to participate in a study conducted in the Department of Biochemistry, Chennai Medical College Hospital & Research Centre, Irungalur, Trichy, Tamilnadu.

Titled - **Estimation of Calcium, Phosphorus, Alkaline Phosphatase and Intact Parathyroid Hormone in various stages of Chronic Kidney Disease.**

Your participation in the study is voluntary

- There will be no cost for participating in the study.
- Your participation is not a compulsion.
- You have the right to withdraw from the study at any time.

Nature of Study:

- ✓ If any abnormalities are identified, you will be informed for further consultation.
- ✓ The results of this study will be kept confidential.

We believe that the results of this study will be beneficial for advancements in medicine & Science. We assure you that we will not use these result for any other purpose.

Consent

I Mr /Mrs / Ms_____
residing at _____
_____on this day _____ after having read the consent form carrying information for the above mentioned study and I hereby give my consent to take 5ml of my blood sample for the purpose of doing Serum Calcium, Phosphorus, Alkaline phosphatase and intact Parathyroid hormone. I was explained about the procedure in detail and give my consent for participating in the study and for using the results for Medical & Scientific purposes.

Signature of the participant

Signature of the Investigator

CONSENT FORM - TAMIL

சென்னை மருத்துவக்கல்லூரி மருத்துவமனை மற்றும் ஆராய்ச்சி மையம்,
இருங்களூர், திருச்சிராப்பள்ளி – 621 105

ஒப்புதல் படிவம்

சென்னை மருத்துவக்கல்லூரி மருத்துவமனை மற்றும் ஆராய்ச்சி மையத்தின் உயிர் வேதியியல் துறையில் நடத்தப்படும்

நாள்பட்ட சிறுநீரக நோயாளர்களில் கால்சியம், பாஸ்பரஸ், அதன்தசைன் பாஸ்பேடஸ் மற்றும் பாராதைராய்டு ஹார்மோன் ஆகியவற்றின் அளவுகளின் ஆய்வு.

பங்கேற்குமாறு உங்களை கேட்டுக் கொள்கிறோம்.

- இப்பரிசோதனைக்கு சம்மதிப்பது உங்கள் விருப்பத்தைப் பொறுத்தது.
- இச்சோதனைக்கு கட்டணம் கிடையாது.
- கட்டாயம் ஏதும் இல்லை.
- பரிசோதனையிலிருந்து எந்நேரமும் விலக தங்களுக்கு முழு உரிமை உண்டு.

இந்த ஆய்வின் முடிவுகள் மருத்துவம் மற்றும் விஞ்ஞான முன்னேற்றத்திற்கு உதவும் என்று கருதுகின்றோம். இவைகளை வேறு எதற்கும் பயன்படுத்தப்பட மாட்டாது என உறுதியளிக்கிறோம்.

ஒப்புதல்

நான் திரு/திருமதி/செல்வி/ _____

முகவரி _____

_____ நாள் _____

அன்று மேற்கண்ட ஆய்வுக்காக தகவல் படிவத்தினை படித்து, கேட்டு புரிந்து கொண்டு இந்த ஆராய்ச்சிக்கு தேவையான சோதனைக்கு என்னிடம் இருந்து 5மிலி. இரத்தம் எடுத்துக் கொள்ள அனுமதிக்கிறேன். என் மனப்பூர்வமான சம்மதத்தை அளிப்பதோடு இந்த ஆய்வின் முடிவுகளை மருத்துவம் மற்றும் விஞ்ஞான நோக்கத்திற்கு பயன்படுத்த ஒப்புதல் அளிக்கிறேன்.

பங்கேற்பாளர் கையொப்பம் ஆய்வாளர் / சம்மதம் பெறுபவர் கையொப்பம்

Master Chart - Cases

S. No.	Age	Sex	CKD Stage	Urea	Creatinine	Calcium	Phosphorus	ALP	PTH
1.	66	M	V	99	11.8	7.2	2.4	54	129
2.	34	M	V	105	8.2	9.5	2.6	69	230
3.	52	F	V	88	5.5	8.2	6.9	84	141
4.	44	F	V	253	9.5	7.5	3.4	95	176
5.	41	M	V	149	8	9.1	5.4	82	362
6.	52	F	V	105	4.8	9.9	5.8	89	244
7.	59	F	IV	76	2.5	8.6	3.7	71	319
8.	71	M	IV	125	2.6	8.8	2.9	78	92
9.	65	M	V	74	7.3	8.9	2.3	144	223
10.	60	M	IV	78	2.5	8.3	2.9	66	71
11.	61	M	V	240	8.1	8.1	2.8	108	258
12.	53	M	V	193	6.9	8.2	3.7	86	337
13.	64	F	V	128	7.3	8.6	5.9	69	94
14.	53	M	V	170	7.9	9.9	6.7	83	92
15.	39	M	IV	24	3.4	8.3	4.3	71	82
16.	51	M	V	190	7.2	9.4	8.1	82	253
17.	65	M	V	160	5.8	6.1	4.2	86	132
18.	58	F	IV	95	3.2	6.9	6.6	97	231
19.	64	F	V	140	10.2	6.7	8.2	138	246
20.	25	F	V	241	14.2	7.6	9.9	345	283
21.	63	M	V	102	10.7	8.4	5.2	99	343
22.	34	M	II	20	1.1	6.9	3.7	154	66
23.	47	M	II	22	1.2	7.3	5.1	106	62
24.	50	F	II	17	0.9	8.1	4.6	84	88
25.	43	M	III	20	1.3	6.4	4.7	136	89
26.	31	M	II	22	1.1	6.1	5.3	81	84
27.	46	F	V	191	15.3	9.1	3.1	96	375
28.	50	M	V	215	15.1	8.2	4.6	109	226
29.	37	M	I	25	0.8	9.2	3.7	87	31
30.	43	M	I	20	0.7	8.8	3.8	74	78
31.	29	F	V	214	9.5	9.6	4.3	62	171
32.	47	F	III	20	1.2	8.9	4.9	65	92
33.	60	M	III	50	1.3	7.2	8.5	58	69
34.	29	F	III	22	1.3	6.8	3.8	93	142
35.	59	M	II	21	1.1	8.7	4.3	57	165
36.	51	M	I	25	0.7	8.3	5.1	94	81
37.	28	M	I	23	0.8	7.1	5.1	76	78
38.	45	M	II	25	1.1	8.1	3.5	97	89
39.	34	M	II	30	1.1	9.6	5.1	76	62
40.	49	M	II	30	0.9	9.8	3.8	49	66
41.	60	M	II	22	1.1	9.4	4.1	101	63
42.	38	F	II	35	0.9	9.9	3.7	64	65
43.	37	M	II	27	0.9	9.5	3.5	67	142
44.	55	M	II	20	1.1	8.3	3.1	64	71
45.	40	M	II	13	0.8	8.7	3.2	88	73
46.	34	M	II	15	1.0	8.3	2.9	71	65
47.	51	M	II	19	0.8	9.1	3.6	62	47
48.	54	F	II	22	0.7	8.5	3.4	64	59
49.	39	M	II	18	0.8	9.2	3.6	71	40
50.	60	M	II	20	0.7	8.7	3.1	74	34

Master Chart - Controls

S. No.	Age	Sex	Urea	Creatinine	Calcium	Phosphorus	ALP	PTH
1.	41	M	23	0.7	9.1	2.4	124	46.1
2.	56	F	18	0.5	8.6	2.7	75	47.2
3.	52	M	28	0.6	9	2.3	85	66
4.	28	M	19	0.7	8.8	2.5	66	32.3
5.	19	F	28	0.5	9.9	3.2	93	59
6.	25	M	18	0.8	8.6	2.8	46	13.7
7.	54	M	32	0.5	8.5	2.9	68	50
8.	43	F	24	0.5	9.8	3.1	90	65
9.	45	F	36	0.7	8.6	3.7	78	50.6
10.	59	M	31	0.6	8.5	3.5	89	27.8
11.	56	M	31	0.6	9.5	3.4	88	27.5
12.	54	M	16	0.6	8.9	3.9	98	15
13.	66	F	24	0.6	9.1	3.5	78	44.8
14.	65	M	26	0.6	9.3	3.4	99	63
15.	51	F	17	0.5	9.1	4.1	123	60
16.	43	F	27	0.6	9.7	2.9	89	99.4
17.	24	M	18	0.7	8.2	4.3	62	54
18.	62	M	23	0.5	7.8	3.1	121	66
19.	21	M	22	0.6	8.1	4.2	94	62
20.	47	F	21	0.4	8.9	3.9	59	50
21.	47	F	15	0.6	7.9	4.1	154	66
22.	50	M	22	0.7	7.1	4.9	93	54
23.	53	M	24	0.6	9.6	3.3	95	60
24.	19	F	30	0.7	9.9	3.1	68	24.5
25.	22	M	18	0.5	8.7	3.8	54	60
26.	26	F	11	0.6	9.4	2.5	85	59
27.	19	M	26	0.5	9.3	3.3	90	56
28.	19	M	23	0.4	9.6	2.9	87	67
29.	27	M	31	0.4	8.2	3.7	63	61
30.	26	M	14	0.5	7.8	3.1	40	57
31.	26	F	17	0.7	8.3	4.2	52	60
32.	55	F	15	0.6	7.7	3.7	52	56.7
33.	27	M	28	0.5	8.9	4.1	94	16
34.	51	M	19	0.7	8.3	4.2	103	62
35.	62	F	39	0.6	9.5	3.9	97	43
36.	52	M	37	0.5	9.3	3.5	98	45
37.	51	M	20	0.7	9.9	2.7	77	54
38.	21	F	29	0.6	9.9	2.5	78	54
39.	45	M	32	0.6	9.7	3.6	66	47
40.	51	M	30	0.4	9.9	2.9	65	34
41.	42	M	15	0.4	9.8	4.1	87	67
42.	25	F	14	0.4	9.9	3.9	70	65
43.	47	M	23	0.5	9.8	3.7	78	66
44.	27	F	20	0.6	9.9	3.8	90	66
45.	42	M	12	0.6	8.6	4.2	66	58
46.	54	M	34	0.5	8.2	4.1	87	57
47.	59	M	26	0.8	9.4	2.6	58	41.2
48.	56	F	19	0.6	8.8	3.5	134	59
49.	58	M	17	0.8	7.1	4.0	155	60
50.	60	F	19	0.6	7.9	2.9	81	110